Acta physiol. scand. 1958. 45. 287—292.

From the Department of Pharmacology, University of Gothenburg, Gothenburg, Sweden.

The Effect of D-Lactic Acid on the Oxygen Consumption and Lactic Acid Content of the Blood in Rabbits.

By

LENNART LUNDHOLM.

Received 12 June 1958.

Abstract.

LUNDHOLM, L. The effect of D-lactic acid on the oxygen consumption and lactic acid content of the blood in rabbits. *Acta physiol. scand.* 1959. *45*. 287—292. — The effect of D- and L-lactic acid on the lactid acid content of the blood and the oxygen consumption was compared in tests on unanesthetized rabbits. The rise in the lactic acid content after intravenous infusion of 11.5 mg D-lactid acid per kg per min for 60 min was twice that after the same dose of the L-isomer. The total increase of the oxygen consumption was about the same after D- and L-lactic acid. It was calculated that at identical blood concentrations the L-isomer stimulated the oxygen consumption about four times stronger than the D-isomer.

The ability of the organism to utilize D-lactic acid seems to have been first investigated by DAKIN and DUDLEY (1913). After oral administration of that substance in a dog with phlorhizin diabetes, they found that an amount of glucose equivalent to that of the lactic acid was excreted in the urine. From this they concluded that the organism was able to utilize D-lactic acid equally well as the isomer L-lactic acid (sarcolactic acid), which occurs naturally therein. However, MEYERHOF and LOHMAN (1926), in *in vitro* experiments, found that addition of D-lactic acid increased

only very slightly the oxygen consumption and glycogen content of isolated frog muscle, whereas addition of L-lactic acid greatly stimulated both respiration and carbohydrate synthesis. In experiments on liver, kidney and brain tissue from rats, the difference between the utilization of the two isomers was even more pronounced. From these experiments the authors concluded that D-lactic acid was scarcely utilized at all by warmblooded animals. CORI and CORI (1928) found that with oral administration of identical amounts of L- and D-lactic acid in rats, the glycogen content of the liver increased four times more after the former than after the latter substance. The lactic acid content of the blood rose much higher in the experiments with D-lactic acid than in those with L-lactic acid. About 30 per cent of the administered D-lactic acid, but none of the L-lactic acid, was excreted in the urine. The authors concluded that utilization of D-lactic acid was four times slower than that of L-lactic acid. Confirmative results were obtained in rabbits (BUCHWALD, CORI and FISHER 1933).

In the course of my own experiments into the relationship between oxygen consumption and lactic acid content of the blood, the question arose as to the degree to which DL-lactic acid could be substituted for L-lactic acid, which is somewhat laborious to produce. A comparative study of the effects of these two optical isomers on the oxygen consumption and blood lactic acid was therefore considered worthwhile.

Method.

The experiments were performed on unanesthetized rabbits which had been deprived of food for 15 hours. Lactic acid in the form of sodium lactate was infused intravenously with an apparatus designed for the purpose. Arterial blood was taken at regular intervals for lactic acid assay *ad modum* FRIEDEMANN, COTONIO and SCHAFFER (1927). The D-lactic acid was prepared with a method reported by IRVINE (1906) and somewhat modified by the writer. Both the experimental methods and preparation of the lactic acids have been described in detail elsewhere (LUNDHOLM 1949, pages 91, 99).

The purity of the D-lactic acid was determined both from the optical activity and from the water content of the crystalline zinc salt. The optically active isomers crystallize with 2 H_2O; the racemic salt with 3 H_2O. Analysis gave the following data:

$Zn(C_3H_6O_3)_2 \cdot 2\ H_2O$. Calculated H_2O 12.88:
found H_2O 13.01

$$\left[\alpha\right]_D^{20} = +\ 6.80 \text{ (c = 3.043, in distilled water).}$$

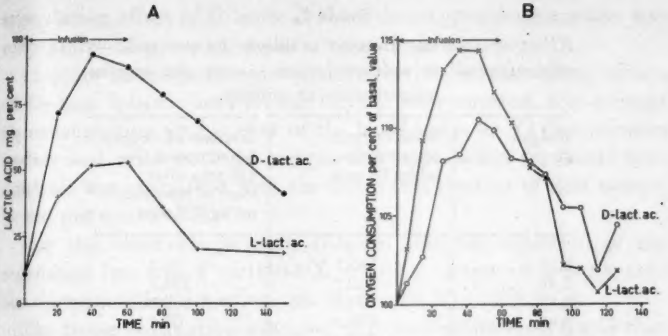

Fig. 1. Effect of infusing 11.5 mg D- and L-lactic acid per kg per min for 60 min on the lactic acid content of the blood (A) and the oxygen consumption (B).

IRVINE (1906) reported + 6.80.
The zink salt of L-lactic acid used in a comparable study (LUNDHOLM 1949) gave following analytical results:

Calculated H_2O 12.88
found H_2O 14.02

$$\left[\alpha\right]_D^{20} = -6.01 \text{ (c = 4.739, in distilled water).}$$

IRVINE (1906) reported —6.83.
The content of D-lactic acid in this preparation estimated from the water content was 88.8 per cent. The sodium lactate was prepared from the zinc salt by conversion with an equivalent amount of NaOH, after which the insoluble zinc hydroxide was filtered away.

Results.

In five rabbits 11.5 mg D-lactic acid per kg/min in the form of sodium lactate was infused for 60 min, after the basal oxygen consumption had first been recorded for 60 min. The oxygen consumption was then measured for 120 min as from the start of infusion. Blood samples for lactic acid assay were taken immediately before and 20, 40, 60, 80, 100 and 150 min after the start of infusion. The results are presented in Fig. 1 and Table I. As will be seen, the lactic acid content of the blood already reached its maximum value, 96 mg per cent, after 40 min, and thereafter remained almost constant during the continued infusion. When the latter had terminated the content fell, in a linear function of time, fairly slowly towards the basal value, which, by extrapolation of the

Table I.

Effect of 11.5 mg D-lactic acid per kg per min — administered as sodium lactate — on the oxygen consumption in rabbits.

Weight kg	Basal oxygen consumption, ml/kg/60 min	Increase of oxygen consumption 0—120 min after start of infusion, ml/kg/120 min
2.55	645.1	55.7
2.38	719.7	108.9
2.65	679.6	167.3
2.90	676.2	58.4
3.15	595.6	57.8
Mean:		89.6 ± 21.8 $P < 0.02$

curve, was estimated to be reached about 220 min after the beginning of the infusion. The effect on the oxygen consumption showed approximately the same course as that on the lactic acid content, increasing maximally to 110 per cent of the basal value at the same time as that content reached its maximum.

Fig. 1 also shows, for comparative purposes, the effect on the oxygen consumption and blood lactic acid in twelve experiments with infusion of a substance containing 88.8 per cent L-lactic acid in a dose averaging 11.5 mg per kg per min. These experiments were reported in an earlier paper (LUNDHOLM 1949, p. 105). The lactic acid content of the blood, as will be noted, rose only half as much as it had done after D-lactic acid. The oxygen consumption, on the other hand, showed a greater increase than that after D-lactic acid, although the lactic acid content of the blood was lower in the experiments with L-lactic acid. It should already be evident from Fig. 1 that D-lactic acid is metabolized more slowly than the L-isomer.

Discussion.

It is evident from Fig. 1 that whereas the increase in the lactic acid content of the blood in tests with D-lactic acid was about double that with L-lactic acid the maximal increase in the oxygen consumption after D-lactic acid was only about 70 per cent of the maximal increase after L-lactic acid. This indicated that the

stimulating effect of D-lactic acid on the oxygen consumption was only about 35 per cent of the effect of L-lactic acid.

In order to compare more quantitatively the stimulating effects of D- and L-lactic acid on the oxygen consumption, the average O_2-consumption (in per cent of the basal value = Y) five minutes before and after a blood sample had been taken for lactic acid analysis was correlated with the lactic acid content of that sample in mg per cent (= X).

For the observations with D-lactic acid the equation of the regression line was $Y = 0.043\ X + 103.1$; in case of L-lactic acid the corresponding equation was $Y = 0.16\ X + 102.7$.

The linear correlation was used as it had before been found that it fitted the experimental values as good as the correlation between oxygen consumption and the logarithm of the lactic acid content (LUNDHOLM 1949). From the ratio of the coefficients of regression it emerged that if the D-lactic acid content of the blood was increased to the same extent as in tests with L-lactic acid the stimulating effect of D-lactic acid on the oxygen consumption was about 3.8 times less than that of the L-lactic acid. That is at the same blood concentration D-lactic acid was metabolized about four times slower than L-lactic acid.

On the other hand the blood lactic acid reached considerably higher values in the experiments with D-lactic acid than in those with L-lactic acid and the duration of stimulated oxygen consumption was longer in the former experiments. The total increase in the oxygen consumption in experiments with D-lactic acid 0—120 min after the infusion had begun was thus of the same magnitude (89.6 ± 21.8 ml per kg per 120 min. Table I.) as in the experiments with L-lactic acid (81.5 ± 8.7 ml, LUNDHOLM 1949).

Summary.

1. The effect of infusing 11.5 mg/kg/min of D-lactic acid on the oxygen consumption and lactic acid content of the blood was investigated in unanesthetized rabbits, and compared with the corresponding effects of L-lactic acid in the same dose.

2. The rise in the lactic acid content of the blood following infusion of D-lactic acid was double that after the same amount of L-lactic acid, and of a longer duration. The stimulating effect of D-lactic acid on the oxygen consumption was at the same blood concentration about four times less than that of L-lactic acid.

3. As the rise in the lactic acid content of the blood was higher and of a longer duration in experiments with D-lactic acid than in those with L-lactic acid the total increase of the oxygen consumption was of the same magnitude after infusing equivalent amounts of the two isomers.

References.

Buchwald, K. W., C. F. Cori and R. E. Fisher, J. biol. Chem. 1933. *103*. 763.
Cori, C. F. and G. T. Cori, J. biol. Chem. 1929. *81*. 389.
Dakin, S. and A. Dudley, J. biol. Chem. 1913. *15*. 143.
Friedemann, Th. E., M. Cotonio and Ph. A. Shaffer, J. biol. Chem. 1927. *73*. 335.
Irvine, J. C., J. chem. Soc. 1906. *89*. I. 317.
Lundholm, L., Acta physiol. scand. 1949. *19*. Suppl. 67.
Meyerhof, O. and K. Lohman, Biochem. Z. 1926. *171*. 421.

Acta physiol. scand. 1959. *45*. 293—299.

From the Department of Ear, Nose and Throat, Karolinska sjukhuset, and the Department of Pharmacology, Karolinska institutet, Stockholm 60, Sweden.

Salivary Secretion in Man Elicited by Means of Stimulation of the Chorda Tympani.

By

H. DIAMANT, B. ENFORS and B. HOLMSTEDT.

Received 6 October 1958.

Abstract.

DIAMANT, H., B. ENFORS and B. HOLMSTEDT. Salivary secretion in man elicited by means of stimulation of the chorda tympani. *Acta physiol. scand.* 1959. *45*. 293—299. — During some operations on the ear in man the chorda tympani is exposed and becomes accessible to experiment. Electrical stimulation caused secretion of saliva, with maximal response at 10 impulses per sec and 2—3 V. Atropine abolished the response. Intravenous infusion of acetyl-β-methylcholine iodide some days after cutting the chorda tympani on one side caused bilateral secretion of saliva while the response to lemon juice on the tongue was greatly reduced on the operated side.

When performing modern surgery for the clinical condition of otosclerosis consisting of a mobilization of the stapes, the middle ear is exposed through the external auditory meatus. All the structures of the middle ear including the chorda tympani become easily accessible in this way. In these cases it is possible to stimulate the chorda tympani and to measure the salivary response quantitatively. Sometimes it is necessary to sacrifice this nerve in order to facilitate the operation. In contrast to the abundant number of

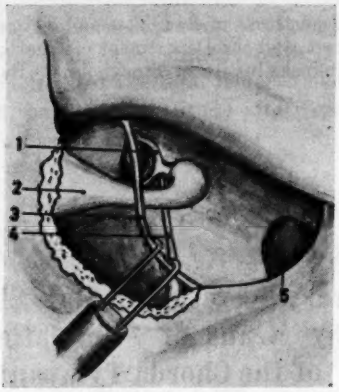

Fig. 1. Application of the electrodes to the intact chorda tympani.

1. Stapes.
2. Incus.
3. Chorda tympani.
4. Stapedius muscle.
5. Fenestra rotunda.

experiments on animals (see EMMELIN 1952) found in the literature we have not come across a study on the stimulation of the chorda tympani in man. The present investigation was undertaken in order to study the salivary secretion in response to electrical stimulation of the chorda tympani. Of particular interest was to investigate the effect of strength and frequency of the electrical stimulation as well as to study to what extent the afferent fibres of the chorda tympani contribute in eliciting the salivary reflexes in response to gustatory stimulation of the tongue.

Method.

The operations were performed under general anaesthesia without premedication with anticholinic drugs. The usual procedure was the following: the patient received one hour before the operation 0.15 g Nembutal per os. A combination of enibomal natr. + phenazone (isopropyl-β-bromallyl-N-methyl barbituric acid) (Narkotal, Astra), nitrous oxide and Suxamethonium (Di-/Trimethylammonium-ethyl/-succinate dichloride "Scoline" Allen & Hanbury) (Celocurin, Vitrum) was used as general anaesthetic. The patient was intubated and breathed through

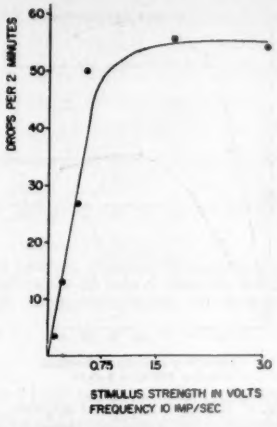

Fig. 2. Effect of increased strength of stimuli on the secretory response to the stimulation of chorda tympani.

a cuffed tube. In prolonged operations with a profuse secretion of saliva intravenous infusion of saline was administered. The secretion of saliva was recorded according to DIAMANT, DIAMANT and HOLMSTEDT (1957) through a plastic cannula inserted into the submandibular ducts on each side. The side not operated upon was used as a control. After incision into the skin of the meatal wall the posterior part of the ear drum was folded forwards. The contents of the middle ear was as a rule fully exposed in this way. Sometimes, however, parts of the bone had to be removed in order to get a proper view of the base of the stapes. The chorda tympani was freed then from the neighbouring tissues. An electrode designed especially for the purpose was applied to the chorda tympani and allowed to remain in position during the course of the experiment (Fig. 1). The nerve was left intact in most of the experiments. In some cases the cut peripheral end was stimulated after stimulation of the intact nerve.

Stimuli were delivered by a Grass Model S 4 Stimulator. Rectangular pulses of 5 millisec duration were used, the frequency ranged from one impulse every other second to 50 impulses per sec. Each stimulation period amounted to 2 min for every given frequency, starting with low frequencies. Two minutes stimulations were chosen with regard to the necessity of obtaining a sufficient number of drops to make the comparison reliable. The voltage of the stimulation varied up to 7 V.

Infusions of acetyl-β-methylcholine iodide (Betacholyl, Vitrum) were given as controls before, during, and after the operation. In some cases complementary controls of secretion were performed using lemon juice on the tongue as a stimulus. After completion of the stimulation, the

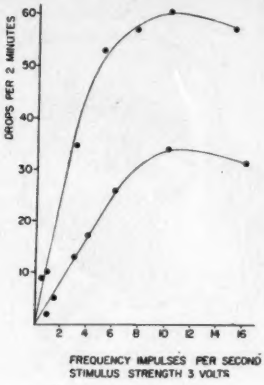

Fig. 3. Effect of increased impulse frequency on secretory response to the stimulation of the chorda tympani.

chorda was divided. The pulse rate was continuously recorded throughout the experiment by means of an electrocardiographic pulse rate recorder.

The experiments lasted approximately one hour and did not in any way influence the following operation or the post-operative course.

Results.

The results of variation in the strength and in the frequency of the stimulus are evident from Fig. 2 and 3. When the frequency was kept at 10 impulses per second and the strength of the stimulus was increased gradually, the response in salivary secretion increased until 2 and 3 V. were reached after which no further increase in response could be elicited (Fig. 2).

In the subsequent experiments the strength of the stimulus was set at 3 V. and the impulses per second were varied systematically. As is seen from Fig. 3 this has brought about an increasing response to a rising frequency up to 10 impulses per sec. Higher frequencies resulted in a slight decrease of the response as evident from the curve. This slight decrease was maintained up to the frequency of 50 impulses per sec, the highest frequency tested. The curves from two patients are illustrated in Fig. 3. The maximal

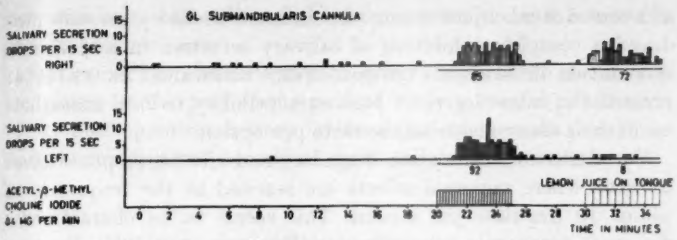
GL SUBMANDIBULARIS CANNULA
SALIVARY SECRETION DROPS PER 15 SEC RIGHT
SALIVARY SECRETION DROPS PER 15 SEC LEFT
ACETYL-β-METHYL CHOLINE IODIDE 04 MG PER MIN
LEMON JUICE ON TONGUE
TIME IN MINUTES

Fig. 4. Comparison of salivary response to an injection of acetyl-β-methylcholine iodide and to the application of lemon juice on the tongue. Bilateral recording with the chorda tympani cut on the left side.

height of the individual response varied considerably in the different patients, but the shape of the curve was always the same. When stimulation was tried after 0.5 mg atropine injected i.v. no secretion could be elicited.

A comparison between the results of stimulation of the intact chorda tympani and of the peripheral end of the cut nerve showed no difference.

In three cases where the chorda tympani had been cut at the operation, the salivary secretion was tested using stimulation with lemon juice on the tongue and MeCh in i.v. infusion. The first test was performed 3—4 days after the operation. Fig. 4 shows a typical result. As it can be seen from this figure there is no sign of increased secretion at rest. Stimulation with MeCh gives an increase on the operated side. Stimulation with lemon juice, on the other hand, gives only a very small response on the operated side. All cases gave exactly the same answer at repeated tests up to four weeks following operation.

Discussion.

In the experiments described, great care was taken to avoid sources of error. Nembutal was used as premedication and preoperative testing of this agent showed it to be without influence on the salivary secretion under the experimental conditions used. In some early experiments the operations were performed during local anaesthesia with lidocaine (1 per cent xylocaine). A dose

of about 1.5 ml injected into the external auditory meatus produced a complete inhibition of salivary secretion in response to stimulation of the chorda tympani. FRENCKNER and PREBER (1954) noticed the same degree of high susceptibility to local anaesthetics in their experiments on the taste perception.

Correlation of stimulation frequency and effector response gives a curve where maximal effects are reached at the frequency of about 10 impulses per second. This seems to be characteristic for most autonomic neuroeffectors (ROSENBLUETH 1950, FOLKOW 1955, CARLSTEN, FOLKOW and HAMBERGER 1957).

The observations with increased frequency of stimulation indicate that impulses between zero and up to 10—12 impulses per sec will cover practically the whole range of obtainable response from the submandibular gland. This suggests that salivary secretion under normal conditions is maintained by relatively low discharge rates, probably 1 or 2 impulses per sec or less. Another possibility would be that only a fraction of the nerve fibres are concomitantly active but they are discharging at higher rates. Recordings of action potentials from the chorda tympani in man may throw further light upon this problem.

The chorda tympani of man contains a few fibres up to 12 or 14 μ diameter, of the sizes carrying touch impulses in peripheral skin nerves, and the main bulk of fibres are delta and B-fibres 4 to 6 μ range. (COSTEN *et al.* 1951.)

Analogies with animal experiments make it likely that the secretory fibres fall within the group of fibres with smaller diameter (ZOTTERMAN 1936, 1953). Direct measurements made on the cat showed that the conduction velocity of the secretory fibres of the chorda tympani was 4—12 m per sec (COHEN *et al.* 1957). The results demonstrated in the curve with increased strength of stimuli supports the view that the secretory fibres are a relatively homogeneous group, since increase in the strength of the stimuli forms a hyperbolic curve suggestive of a steady recruitment of new fibres. The complete agreement between the result obtained in stimulation of the intact and the cut chorda tympani may have many explanations. Probably this is caused by a central depression of the salivary reflex by the general anaesthesia.

The experiments with mecholyl and lemon juice demonstrated in Fig. 4 make it suggestive that ipsilateral afferents are of decisive importance for secretory response, since lemon juice on the tongue produces a normal response on the normally in-

nervated side, but only a feeble response on the side where the chorda had been cut.

Cutting of the chorda tympani gives very seldom rise to any complaint on the part of the patient. Only a careful penetration of the post-operative history in these patients reveals sometimes disturbances in taste, mostly of slight and passing nature.

References.

Carlsten, A., B. Folkow and C.-A. Hamberger, Cardiovascular effects of direct vagal stimulation in man. *Acta physiol. scand.* 1957. *41.* 68.

Cohen, M. J., S. Landgren, L. Ström and Y. Zotterman, Cortical reception of touch and taste in the cat. *Acta physiol. scand.* 1957. *40.* 3. Suppl. 135.

Costen, J. B., M. H. Clare and G. H. Bishop, The transmission of pain impulses via the chorda tympani nerve. *Ann. Otol. Rhin. Laryng.* 1951. *60.* 591.

Diamant, B., H. Diamant and B. Holmstedt, The salivary secretion in man under the influence of intravenously infused acetyl-beta-methylcholine iodide. *Arch. int. Pharmacodyn.* 1957. *111.* 86.

Emmelin, N., "Paralytic secretion" of saliva. An example of supersensitivity after denervation. *Physiol. Rev.* 1952. *32.* 21.

Folkow, B., Nervous control of the blood vessels. *Physiol. Rev.* 1955. *35.* 629.

Frenckner, P. and L. Preber, The effect of electric stimulation of the chorda tympani. *Acta oto-laryng.* (Stockh.) 1954. *116.* Suppl. 100.

Rosenblueth, A., The transmission of nerve impulses at neuroeffector functions and peripheral synapses. New York. Technology. Press & Wiley. 1950.

Zotterman, Y., Specific action potentials in the lingual nerve of cat. *Skand. Arch. Physiol.* 1936. *75.* 105.

— Thermal receptors. *Ann. Rev. Physiol.* 1953. *15.* 357.

Acta physiol. scand. 1958. *45*: 300—307.

From the Department of Physiological Chemistry, University of Lund, Lund, Sweden.

Excretion and Nature of Bile Acids Formed from Cholic Acid-24-^{14}C and Deoxycholic Acid-24-^{14}C in Bile Duct Ligated Rats.

By

ARNE NORMAN and BENGT SAMUELSSON.[1]

Received 11 October 1958.

Abstract.

NORMAN, A. and B. SAMUELSSON. Excretion and nature of bile acids formed from cholic acid-24-^{14}C and deoxycholic acid-24-^{14}C in bile duct ligated rats. *Acta physiol. scand.* 1959. *45*. 300—307. — Cholic acid-24-^{14}C and deoxycholic acid-24-^{14}C have been given intraperitoneally to rats at different times after ligation of the common bile duct. Chromatographic separations of the labelled urinary products show the appearence of free bile acids, the amount increasing with the duration of obstruction. Deoxycholic acid is almost completely 7-α-hydroxylated even after longstanding obstruction when the conjugation power has been reduced.

Previous studies of this series have demonstrated that cholic acid and chenodeoxycholic acid are the main bile acids in the rat (BERGSTRÖM and SJÖVALL 1954) appearing mainly as taurine conjugates. Only 1—5 per cent is present as glycine conjugates but no free bile acids have been found in the bile (NORMAN 1954). Metabolic studies of different ^{14}C-carboxy-labelled bile acids in

[1] Present address: Department of Chemistry, Karolinska Institutet, Stockholm 60.

bile fistula rats have shown that cholic acid is a metabolic end product (BERGSTRÖM, ROTTENBERG and SJÖVALL 1953) while chenodeoxycholic acid is transformed into two trihydroxycholanic acids (BERGSTRÖM and SJÖVALL 1954, MAHOWALD *et al.* 1957). The presence of an active system capable of introducing a 7-*α*-hydroxyl group into deoxycholic acid has been demonstrated both *in vivo* (BERGSTRÖM *et al.* 1953) and *in vitro* with rat liver homogenates (BERGSTRÖM and GLOOR 1955). In intact rats the bile acids are eliminated only by fecal excretion. The half-life time of a bile acid molecule in the pool has been estimated to be 2—3 days (LINDSTEDT and NORMAN 1956).

It is well known that biliary obstruction causes bile acids to appear in the urine (for literature see SOBOTKA 1937, 1943). The excretion of ^{14}C-carboxy-labelled deoxycholic, chenodeoxycholic and hyodeoxycholic acid in surgically jaundiced rats has been studied by DOISY *et al.* (MAHOWALD *et al.* 1957 and MATCHINER *et al.* 1957). They showed that the urine is the only excretory pathway and that the same metabolites occur in the urine of these rats as appear in the bile of fistula rats.

The aim of the present investigation is to study the conjugation and 7-*α*-hydroxylation in bile duct ligated rats after varying duration of the obstruction. Cholic acid-24-^{14}C and deoxycholic acid-24-^{14}C were injected intraperitoneally and the labelled excretory products in the urine chromatographically separated and identified.

Experimental.

Cholic acid-24-^{14}C (250,000 cpm/mg) and deoxycholic acid-24-^{14}C (450,000 cpm/mg) were prepared according to BERGSTRÖM, ROTTENBERG and VOLTZ (1953).

Bile duct ligated rats. In these experiments 200—250 g male white rats of the institute stock were used. Bile duct ligation was made under ether anesthesia. The common bile duct was ligated as near the liver as possible by double ligatures, and the interjacent part of the duct was cut. The rats were then placed in metabolism cages with free supply of a normal diet consisting mainly of oats and bread. At different time after ligation, 0.5—1.0 mg of the sodium salt of the ^{14}C labelled bile acids was injected intraperitoneally. Urine and feces were collected daily in 80 per cent ethanol for three days. After this time the rats were killed and the presence of the obstruction controlled at autopsy. The liver with the distended bile ducts and the intestine were removed, homogenized and extracted. In some experiments the rats were kept in desiccators and the expired carbon dioxide

Table I.

Excretion of ^{14}C in bile duct ligated rats after administration of cholic acid-24-^{14}C.

Rat no.	C 1	C 2	C 3	C 4	C 5
Duration of biliary obstruction prior to administration of cholic acid-24-^{14}C	1 day	2 days	5 days	5 days	10 days
Per cent of total dose recovered in:					
Urine (0—24 hours)	43	87	87	74	51
Urine (24—48 hours)	35	2	8	1	29
Urine (48—72 hours)	1	1	1	1	1
Feces (0—72 hours)	1	1	1	1	1
Total	78	89	95	75	80

was collected in sodium hydroxide and assayed for ^{14}C as barium carbonate.

Extraction and fractionation. The feces, the urine and the organs were extracted with 80 per cent ethanol. Aliquots of the extracts were mounted on aluminium planchets for determination of radioactivity. The ethanol extract was evaporated to dryness and the residue dissolved in water, acidified and extracted with butanol.

Separation of the labelled products was performed with the reversed phase partition chromatographic technique described by BERGSTRÖM and SJÖVALL (1951), SJÖVALL (1953) and NORMAN (1953). The following phase systems were used.

Phase system	Moving phase (v/v)	Stationary phase (v/v)
F	Methanol/water 55/45	Chloroform/heptane 9/1
C	Methanol/water 50/50	Iso-octanol/chloroform 1/1
D	Water	n-Butanol

4 ml of the stationary phase was used per 4.5 g of hydrophobic Supercel. Each fraction was titrated with 0.02 N sodium hydroxide in methanol, and aliquots were transferred to copper planchets for determination of the radioactivity.

The separated cholic acid was identified by isotope dilution. The labelled material was diluted with the unlabelled reference substance and the specific activity determined after several recrystallizations.

Results.

Excretion of isotope in bile duct ligated rats after injection of cholic acid-24-^{14}C.

Cholic acid-24-^{14}C was injected intraperitoneally into rats at different times after ligation of the common bile duct. The daily

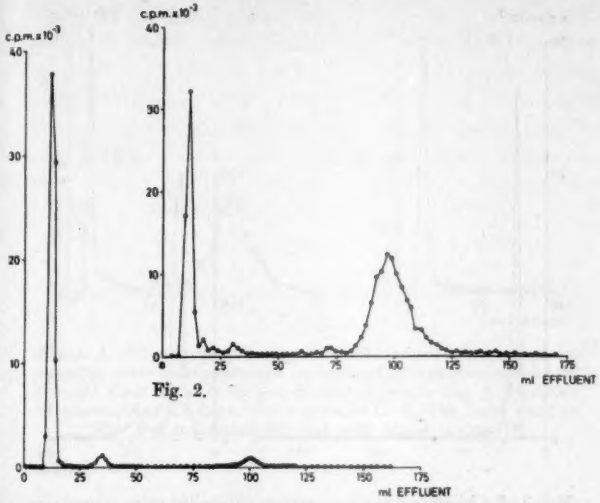

Fig. 2.

Fig. 1.

Fig. 1. Chromatographic separation of labelled urinary products excreted after intraperitoneal injection of cholic acid-24-^{14}C into a bile duct ligated rat. Duration of obstruction: 2 days. Phase system: C.

Fig. 2. Chromatographic separation of labelled urinary products excreted after intraperitoneal injection of cholic acid-24-^{14}C into the same rat as in the experiment shown in Fig. 1. Duration of obstruction: 12 days. Phase system: C.

excretion of isotope in urine and feces was determined and the results of these experiments are given in Table I. The feces contained no radiocarbon. In some animals the activity in the expired carbon dioxide was determined. In no case could any significant amount be demonstrated. The urine was found to be the only excretory pathway for the bile acids in bile duct ligated rats. Most of the isotope was excreted in the urine within the first day and no isotope could be demonstrated after two days. The rate of excretion was not influenced by the duration of the obstruction.

Three days after injection the rats were killed. At autopsy the liver with the distended bile ducts was removed, homogenized and extracted. In most of the animals the extract contained no isotope. In one rat with a longstanding obstruction (3 weeks), however, the bile ducts were found to be exceptionally distended and more than one half of the injected amount was retained in the liver and the ducts.

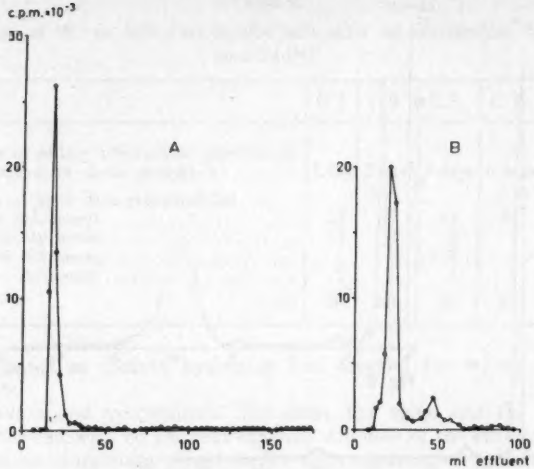

Fig. 3. A. Chromatographic separation of labelled urinary products excreted after intraperitoneal injection of deoxycholic acid-24-^{14}C into a bile duct ligated rat.
Duration of obstruction: three days. Phase system: C.
B. The front band in Fig. 3 A rechromatographed with phase system D.

The nature of the isotope labelled bile acids in the urine after administration of different 24-^{14}C labelled bile acids.

A. Cholic acid. The unhydrolyzed bile acids from the urine of bile duct ligated rats have been chromatographically separated. Fig. 1 gives a chromatographic separation of labelled urinary products from a rat with the bile duct ligated two days prior to administration of cholic acid-24-^{14}C. More than 90 per cent of the labelled acids were found in the front band containing the taurine conjugates, 2—3 per cent were eluated at the place of glycocholic acid (30—40 ml effluent) and 5 per cent at the place of free cholic acid (80—110 ml effluent). The first band was rechromatographed with phase system D and the labelled material appeared at the place of unlabelled taurocholic acid added as a carrier. Fig. 2 shows a chromatographic separation of labelled material in the urine after a new injection of cholic acid-24-^{14}C to the same rat after a further 10 days of obstruction. Now more than 50 per cent of the labelled material was found to be free cholic acid.

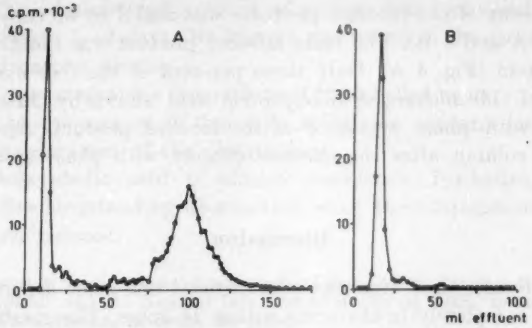

Fig. 4. A. Chromatographic separation of labelled urinary products excreted after intraperitoneal injection of deoxycholic acid-24-^{14}C into the same rat as in the experiment shown in Fig. 3. Duration of obstruction: 14 days. Phase system: C. B. The front band in Fig. 4 A rechromatographed with phase system D.

Similar experiments with three other animals also showed an almost complete conjugation of the bile acids excreted in the urine after two days of obstruction. After ten days obstruction an increased excretion of unconjugated bile acids was observed. There was a great variation in the ratio of free and conjugated bile acids between different animals. 2—4 per cent of the excreted bile acids were found to be conjugated with glycine.

B. Deoxycholic acid. The extent of hydroxylation and conjugation of deoxycholic acid was followed in the same rat at different times after ligation of the common bile duct by chromatographic analyses of the labelled urinary products. The result of one of the experiments is given in Fig. 3 and 4. Chromatography with phase system C separated the taurine conjugates, glycocholic acid, cholic acid and glycodeoxycholic acid. Deoxycholic acid was retained in the stationary phase. After three days of obstruction practically all the activity was found in the taurine conjugate band (Fig. 3 A). Less than 2 per cent of the isotope was found in the eluated stationary phase after the chromatography. The chromatography of the front band with phase system D (Fig. 3 B) showed that the main labelled compound appeared at the place of taurocholic acid. After hydrolysis of the "taurocholic acid band" the unconjugated labelled bile acid was identified as cholic acid. Only small amount of labelled compound was present at the place of taurodeoxycholic acid. After a further 11 days of obstruction, however, only

30 per cent of the labelled products was found to be conjugated (Fig. 4 A and 4 B). The main labelled product was identified as cholic acid (Fig. 4 A). Only three per cent of the free bile acids consisted of unchanged deoxycholic acid shown by chromatography with phase system F of the labelled products remaining in the column after the chromatography with phase system C.

Discussion.

The results show that the administered bile acids are excreted almost completely in the urine within 24 hours. The greater part is conjugated after an obstruction of short duration. It is difficult to decide whether the occurrence of free acids in the urine is caused by injury to the liver cells or whether the bile acids have bypassed the liver circulation and been excreted directly in the urine. The experiments with deoxycholic acid show that the 7-*α*-hydroxylation is almost complete even after longstanding obstruction while the excreted free bile acids increase with the time of obstruction, indicating that the conjugating power of the obstructed liver has decreased. The increased amount of free bile acids after longstanding obstruction also points in this direction. It is not proved, however, that the liver is the only organ capable of 7-*α*-hydroxylation.

Bergström and Gloor (1954, 1955) have demonstrated that conjugation and 7-*α*-hydroxylation take place in rat liver slices and homogenates after incubation with deoxycholic acid-24-^{14}C. Their experiments further indicated that *in vitro* the reaction proceeds from deoxycholic acid via taurodeoxycholic acid to taurocholic acid. In surgically jaundiced rats the 7-*α*-hydroxylation may occur without concomitant conjugation since free cholic acid is excreted in the urine after administration of free deoxycholic acid. It has previously been shown that no hydrolysis of conjugated bile acids occurs in the liver (Norman 1955, Lindstedt and Norman 1957).

Summary.

1. Cholic acid-24-^{14}C and deoxycholic acid-24-^{14}C were given intraperitoneally to rats at different time after ligation of the common bile duct.

2. The administered amount of isotope was recovered in the urine within 1—2 days. No isotope was found in the feces or the expired carbon dioxide.

3. Chromatographic separations of the labelled urinary products show the appearance of free bile acids, the amount increasing with the duration of the obstruction.

4. Deoxycholic acid is almost completely 7-α-hydroxylated even after longstanding obstruction when the conjugation power has been reduced.

This work is one in a series of investigations supported by "Statens Medicinska Forskningsråd" and the National Institutes of Health (H 2842), United States Public Health Service, Bethesda, Maryland.

References.

BERGSTRÖM, S. and U. GLOOR, *Acta chem. scand.* 1954. *8.* 1373.
BERGSTRÖM, S. and U. GLOOR, *Acta chem. scand.* 1955. *9.* 34.
BERGSTRÖM, S., M. ROTTENBERG and J. SJÖVALL, *Hoppe-Seyl. Z. physiol. Chem.* 1953. *295.* 278.
BERGSTRÖM, S., M. ROTTENBERG and J. VOLTZ, *Acta chem. scand.* 1953. *7.* 481.
BERGSTRÖM, S. and J. SJÖVALL, *Acta chem. scand.* 1951. *5.* 1267.
BERGSTRÖM, S. and J. SJÖVALL, *Acta chem. scand.* 1954. *8.* 611.
LINDSTEDT, S. and A. NORMAN, *Acta physiol. scand.* 1956. *38.* 121.
LINDSTEDT, S. and A. NORMAN, *Acta chem. scand.* 1957. *11.* 414.
MAHOWALD, T. A., J. T. MATSCHINER, S. L. HSIA, E. A. DOISY, Jr., W. H. ELLIOTT and E. A. DOISY, *J. biol. Chem.* 1957. *225.* 795.
MAHOWALD, T. A., J. T. MATSCHINER, S. L. HSIA, R. RICHTER, E. A. DOISY, Jr., W. H. ELLIOTT and E. A. DOISY, *J. biol. Chem.* 1957. *225.* 781.
MATSCHINER, J. T., T. A. MAHOWALD, S. L. HSIA, E. A. DOISY, Jr., W. H. ELLIOTT and E. A. DOISY, *J. biol. Chem.* 1957. *225.* 803.
NORMAN, A., *Acta chem. scand.* 1953. *7.* 1413.
NORMAN, A., *Acta physiol. scand.* 1954. *32.* 1.
NORMAN, A., *Proc. roy. Physiographic Soc., Lund.* 1955. 25. No 2.
SJÖVALL, J., *Acta physiol. scand.* 1953. *29.* 232.
SOBOTKA, H., Physiological chemistry of the bile. Baltimore. 1937.
SOBOTKA, H. and E. BLOCH, *Ann. Rev. Biochem.* 1943. *12.* 45.

Acta physiol. scand. 1959. *45*. 308—310.

From the University Institute for Experimental Medical Research, Ullevaal Hospital, Oslo, Norway.

Notes on Rectal Temperature Variations in Dogs During Nembutal Anaesthesia.

By

JOHN KROG.

Received 17 October 1958.

Abstract.

KROG, J. Notes on rectal temperature variations in dogs during Nembutal anaesthesia. *Acta physiol. scand.* 1959. *45*. 308—310. — The body temperature of 25 mongrel dogs under Nembutal anaesthesia was recorded at different environmental temperatures. It was found that at a temperature of about 20—25° C smaller dogs had a tendency to cool off, while larger had a tendency to warm up. The findings are tentatively being explained as being due to impairment of the heat dissipation system of the animals during anaesthesia.

It has been a general experience to find a decline in body temperature in dogs under the influence of Nembutal anaesthesia.

ESSEX (1952) reported that the body temperature in dogs under the influence of adrenaline and Nembutal varies according to body size. Dogs heavier than 15 kg tended to increase the body temperature, whereas small dogs tended to cool down.

In view of the extensive use of dogs in physiological experiments further investigation of these interesting findings seems warranted.

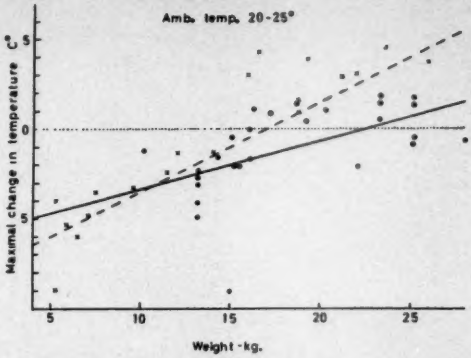

Fig. 1. The maximal deviation in body temperature from pre-anaesthetic level in a number of dogs of different body size anaesthetized with Nembutal sodium and exposed to room temperature of 20—25° C, plotted together with values obtained by Essex.

Crosses indicate the values by Essex, filled dots, own observations. The regression line, (solid) for own observations is calculated according to the least squares method.

The correlation coefficient is 0.57. This coefficient is significant to the 1 % level.

Material and Methods.

25 adult, mongrel dogs were used for the experiments, ranging in body weight from 10—28 kg. Rectal temperature was measured in a temperature controlled room at ambient temperatures ranging from 10—30° C at 5° intervals. Each dog was exposed to this environment for a period of 3—4 hours. The amount of anaesthetic given was 25 mg/kg body weight and additional doses given if shivering or other signs of arousal appeared. During the experiments the rectal temperature was recorded continuously with an accuracy of about $\pm$ 0.2° C, by means of a Brown Honeywell automatic temperature recorder.

Results.

Fig. 1 shows the rectal temperature recorded in our experiments at an environmental temperature ranging from 20—25° C compared with those obtained by Essex during his investigations of the relation between body temperature and tolerance to massive doses of adrenaline in dogs. It appears from Fig. 2 that the change in body temperature (average $\pm$ 2° C for all observations) is related to body size.

Fig. 2 shows that with decreasing environmental temperatures, all dogs had a tendency to cool, also the body temperatures of the larger dogs would fall when the ambient temperature was

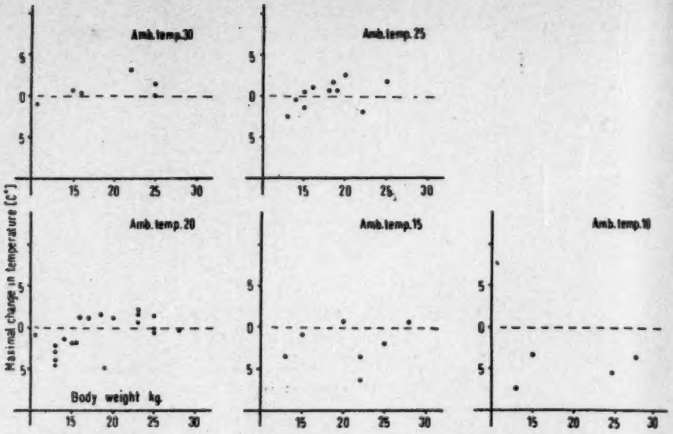

Fig. 2. Maximal deviation from pre-anaesthetic levels in body temperature of dogs after 3 hours when anaesthetized with Nembutal, and exposed to different environmental temperatures.

below 20° C. On the other hand, when the temperature was raised from 25 to 30° C, some of the smaller dogs that cooled off at 20—25° C would show an increase in body temperature.

The results here presented are principally in agreement with the results of ESSEX. The effects of adrenaline may explain why the regression line is somewhat steeper in his experiments than in those here presented. The dependency upon body size of both ours and ESSEX's dogs in their ability to maintain normal body temperature at a certain environmental temperature during anaesthesia, seems to indicate that the found temperature changes are due to disturbances in the heat dissipation system of the animals.

The findings suggest that a continuous check on body temperature is a necessity in conjunction with most physiological experiments in anaesthetized dogs. It also shows that in dogs hypothermia as well as hyperthermia may ensue following anaesthesia with Nembutal.

Reference.

ESSEX, HIRAM E., *Amer. J. Physiol.* 1952. *171*. 1.

Acta physiol. scand. 1959. 45. 311—327.

From the Department of Physiology, Karolinska Institutet, Stockholm 60, Sweden.

Determination of Cholinesterase in the Cellular Components of Neurones.

By

EZIO GIACOBINI.

Received 7 November 1958.

Abstract.

GIACOBINI, E. Determination of cholinesterase in the cellular components of neurones. *Acta physiol. scand.* 1959. *45*. 311—327. — The procedure for the determination of cholinesterase activity in $\mu\mu$l samples of cytoplasm and nucleoplasm of isolated nerve cells is described. The method is an application and modification of the cartesian diver technique of LINDERSTRÖM-LANG, which allows quantitative determinations in the order of 1×10^{-6} μl CO_2/hour with $\pm$ 15 % accuracy. By means of this method acetylcholinesterase activity could be demonstrated in the cytoplasm and the nucleus of anterior horn cells of the spinal cord of the rat.

The most commonly used method for studying the intracellular localization of enzymes in recent years has been the technique of differential centrifugation (BENSLEY and HOERR 1934, CLAUDE 1946, HOGEBOOM, SCHNEIDER and PALADE 1947—1948, SCHNEIDER and HOGEBOOM 1951). This method suffers from several disadvantages (ALLFREY, MIRSKY and STERN 1955, BRADFIELD 1950, GLICK 1953, HOLTER 1952, HOLTER and LINDERSTRÖM-LANG 1951,

KOPAK 1950—1955, NOVIKOFF 1956, NOVIKOFF *et al.* 1953, SCHNEIDER and HOGEBOOM 1956), the most serious being the inhomogenity of the preparation in respect to cell type and the difficulty of securing a sharp separation of the various subcellular fractions by centrifugation alone.

These disadvantages could be avoided if the various subcellular structures were first separated by microdissection and the enzyme activity of these structures determined by sufficiently sensitive methods. For most enzymes such methods do not presently exist, but in the case of the cholinesterase[1] (ChE) two methods are available, one quantitative and one qualitative, with the necessary degree of sensitivity. These are the present micromodification of the cartesian diver technique (LINDERSTRÖM-LANG 1937, ZAJICEK and ZEUTHEN 1956, ZEUTHEN 1953) which permits quantitative estimation on $\mu\mu$l quantities of nucleo- and cytoplasm, and the histochemical thiocholine method of KOELLE and FRIEDENWALD (1949).

In a previous investigation (GIACOBINI 1956) attempts were made to study the intracellular distribution of ChE by applying the histochemical method on single cells and their parts isolated by microdissection from various regions of the nervous system. In neurones belonging to the spinal, sympathetic and parasympathetic ganglions as well as in the anterior and lateral horn cells, a deposition of copper thiocholine crystals inside and over the surface of the cell body and the axon indicates the presence of the enzyme. From these preparations no exact localization was considered to be possible considering the artefacts produced by the method. Positive histochemical tests were also obtained from isolated portions of the cytoplasm having a surface of about 15 μ^2 after an incubation time of 15—30 min. Isolated nuclei observed after the same incubation time did not show any positive reaction.

Prolonging the incubation time to about 2 $^1/_2$—3 h it was possible to demonstrate ChE activity in single nuclei preparations (see Fig. 3, C and D).

In the present communication a technique is described for the determination of ChE and AChE in small amounts of different

[1] Abbreviations: ChE = all enzymes splitting choline esters, irrespective of their specificity for certain substrates. AChE = acetylcholinesterase, specific cholinesterase, true cholinesterase, erythrocytes cholinesterase ("e"type), acetocholinesterase, Cholinesterase I. Non spec. ChE = non specific cholinesterase, pseudocholinesterase, serum cholinesterase ("s"type), butyro- and propionocholinesterase, Cholinesterase II.

cellular components isolated from single nerve cell preparations, having a volume in the order of magnitude of 0.1—0.01 $\mu\mu$l (1 $\mu\mu$l = 10^{-6} μl).

Preliminary results of this investigation have been previously reported (GIACOBINI 1957 b).

Material and Methods.

Microdissection and micromanipulation technique.

All experiments were performed on large anterior horn cells from the cervical tract of the rat spinal cord.

Albino rats weighing about 200 g were killed by decapitation after light ether anaesthesia, the cervical tract of the spinal column was removed and the cord exposed by cutting the vertebral laminae. The cervical tract of the spinal cord was then quickly dissected out and placed in Ringer solution or in a balanced salt solution.

An isolated segment (C_5, C_6 or C_7) about 6—700 μ thick was cut out and placed in the microdissection chamber. The cord was cut with microscissors under microscopical control in 4 cross linked sectors. From the anterior sectors containing the anterior and lateral horn a part containing only the anterolateral region of the anterior horn was further removed. A small fragment of this tissue was then placed in a moist oxygenated chamber containing a Ringer bicarbonate-substrate solution. The separation of the single cells was first performed with the aid of thin metal needles under a magnification of about 120 × and then continued with Zeiss micromanipulators carrying glass needles about 10 μ thick under a magnification of 450 × (GIACOBINI 1956).

In such preparations large and small nuclei were found together with a few entire nerve and glial cells of various dimensions. The morphological structure of these nuclei was well conserved, their nucleoplasm being slightly granular, the nucleolus was visible and the cellular membrane often free from cytoplasmic material. (See Fig. 3 A and B.)

A single cell or a single nucleus was then transferred to a drop of paraffin oil by means of a micropipette. The small amount of the Ringer bicarbonate medium transferred together with the cell was in this way prevented from evaporation. The neurones or their nuclei kept in this way at room temperature generally retain their normal appearance up to more than 24 h.

The isolated nuclei were then carefully inspected under high magnification in phase contrast in order to detect small cytoplasmic particles eventually sticking to the nuclear membrane. The cells or the nuclei which by microscopical inspection were found to be injured in their morphological aspect were discarded. In some preparations a segment 50—100 μ long, of the proximal part of the neurite or of a dendrite, was cut out and transferred to a drop of paraffin oil. In an unstained preparation it is possible to distinguish the initial non medullated portion

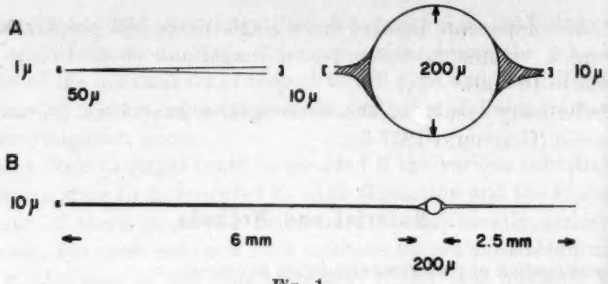

Fig. 1.

A and B: Schematical drawings of the diver micropipette at two different magnifications.

of the axon of anterior horn cells from the soma and the dendrites by the absence of Nissl substance and pigment granules.

A diver of the same type as shown in Fig. 1, was used to aspirate intracellular material from the isolated cell preparation and nuclear material from the single nuclei (see Fig. 3 F).

The samples of nucleoplasm were obtained from the entire isolated cell, from occasionally isolated nuclei and finally from nuclei, isolated by means of micromanipulation. Only single cell or single nucleus preparations were used (see Fig. 3 A, B and F). In some experiments the nucleolus could first be isolated and successively aspirated in the diver. The samples of cytoplasm and nucleoplasm employed in these experiments ranged from about 0.1 to 0.01 $\mu\mu$l.

Measurement of cellular and nuclear volume.

Micklewright, Kurnick and Hodes (1953) have recently demonstrated that the previously applied formulae for the measurement of nerve cell volume which consider that the cell is a prolate or an oblate spheroid are unreliable. In this investigation the following formula suggested by the mentioned authors has been applied for the calculation of the volume of the cell and nucleus:

$$V = \pi/6\ abc$$

where:

V = cell or nucleus volume
a, b = cross sectional diameters
c = thickness of the cell or of the nucleus.

The linear relationship found from the above mentioned authors between nuclear and cellular volume in the anterior horn cells of cat could be confirmed also for the rat (mean cell volume $= 10.6 \pm 0.9 \times$ nucleus volume). The volume of the segment of dendrites and axons employed for the ChE activity determination was calculated considering the nerve processes as a cylinder.

The volume of the cytoplasmic and nuclear samples was calculated by assuming that the tip of the diver was a regular cylinder. The volume of the inflow of the material was determined by following with a micro-ocular eyepiece the movement of a paraffin drop previously introduced into the capillary. Controls made with the help of coloured substances demonstrated, however, that the error inherent in this technique was about 15 %. The values obtained in this work for the enzyme activity in samples of cytoplasm and nucleus of about 0.1—0.01 $\mu\mu$l has therefore no absolute quantitative significance. A more exact evaluation of such small amount of fluid aspirated from single cells can be reached by means of a volumetric submicromanipulator of the type described by KOPAC (1955) and KOPAC and HARRIS (1951).

Control experiments were performed measuring the dimensions of nuclei and cells which had been kept in the Ringer substrate-bicarbonate solution during 1—3 and 6 h. These experiments demonstrated that the volume of the cells or of the isolated nuclei did not essentially change after 6 h.

Cartesian diver technique.

The cartesian diver technique of LINDERSTRÖM-LANG (1937) was employed in this work. In 1938 LINDERSTRÖM-LANG and GLICK developed a cartesian diver technique for the measurement of cholinesterase based on the principle utilized in the Warburg apparatus (AMMON 1933). This technique is based on the fact that during the enzymatic scission of choline esters in the presence of a bicarbonate buffer the acid liberated will cause an evolution of carbon dioxide which can be measured gasometrically. Essentially, the principle of the diver technique is that any change in the amount of gas contained in a vessel (the diver) requires a corresponding change in pressure to maintain the gas volume constant in order to keep the vessel floating in a medium at a fixed level. In other words, the pressure changes may be taken as a measurement of the changes in the amount of gas in the diver.

The diver apparatus and the technique employed in this work is in principle identical with that originally described and used by ZEUTHEN in 1953 for measurement of respiration of single cell cultures of Tetrahymena piriformis (ZEUTHEN 1953). It has recently been modified by ZAJICEK and ZEUTHEN (1956) for the determination of the ChE activity of single megacaryocytes and used by GIACOBINI and ZAJICEK (1956) and GIACOBINI (1957 a, 1959) for the determination of AChE and ChE in single nerve cells isolated from sympathetic and spinal ganglions and by GIACOBINI and HOLMSTEDT (1958) in single anterior horn cells. For the purpose of this investigation a special type of diver had to be used and the sensitivity of the method to be improved. The theoretical aspects of cartesian diver micromanometry have been treated in earlier papers (LINDERSTRÖM-LANG 1937, LINDERSTRÖM-LANG and GLICK 1938, ZAJICEK and ZEUTHEN 1956, ZEUTHEN 1950, 1953, 1955).

A picture of the cartesian diver assembly used in this work allowing four simultaneous ChE determinations is shown in Fig. 4 A, B. The

diver equipment consists (Fig. 4 A from the left) of a manometer for the macroadjustments of the flotation pressure of the diver, and four "sensitive manometers" (ZEUTHEN 1953, 1955) having a capillary burette volume per mm of about 0.25 μl, connected to the flotation vessels and immersed in a thermostatic bath adjusted at 27° $\pm$ 0.002°.

The picture shows also three horizontal microscopes with ocular scales for the observation of the diver.

For a detailed description of this apparatus the reader is referred to the papers of ZEUTHEN (1950, 1953, 1955).

The gas exchanged per unit time $\Delta CO_2/\Delta t$ was calculated according to a simplification of the formula previously reported (GIACOBINI 1957 a, ZAJICEK and ZEUTHEN 1956, ZEUTHEN 1953) to which the reader is referred.

$$\frac{\Delta\ CO_2}{\Delta\ t} = 4.26 \times 10^{-13}\ (B + h - 360)\ (273\ V_g + 216\ V_f)\ x$$

where:

x = movements in mm per unit time in the capillary micromanometer (1 mm = 0.25 μl)
v = volume in μl per mm of the capillary micromanometer = 0.25
V = volume of the gas phase of the micromanometer in μl = 190,000
V_g = gas volume of the floating diver
B = barometric pressure expressed in mm H_2O (760 mm $\sim$ 10,300 mm H_2O)
h = initial equilibrium pressure of the diver read on the big manometer.
e = vapour tension of water in mm H_2O at 27° (= 360 mm H_2O)
$\alpha\ CO_2$ = absorption coefficient for CO_2 at 27° (= 0.721)
V_f = volume of the fluid enclosed in the diver in μl.

The volume of the gas in the floating diver (V_g) may be measured with reasonable accuracy, as the gas bubble contained in it is practically a sphere.

Two determinations of the diameter of the gas bubble were done, first with the ocular microscope (see Fig. 4 A, B and D) at the beginning of the experiment when the diver was already floating in the vessel, and then at the end of the experiment using higher magnification with a standard microscope supplied with an ocular eyepiece. The volume of the gas bubble was calculated by the mean value of these two diameters.

The volume of the liquid charge (V_f) of the diver can be computed by subtracting the weight of the diver after drying from the weight of the diver at the end of the experiment. However, owing to the difficulty of weighing with accuracy such a small quantity of fluid, it was found more convenient to calculate the volume directly by the dimensions of the diver.

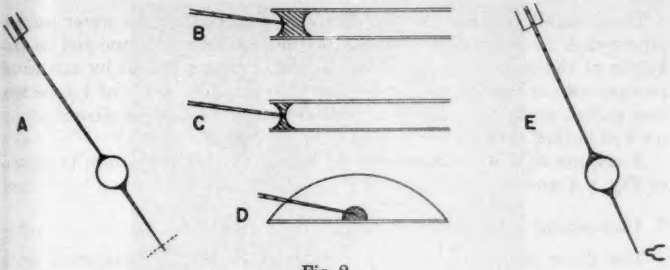

Fig. 2.

A: The tip of the diver micropipette is broken at its end under microscope control.

B: The open extremity is introduced in a capillary having a constant diameter (10—20 μ) and containing a small quantity of the inhibitor previously introduced by means of a micropipette. The volume of this fluid is calculated by the dimensions determined with a micrometer eyepiece.

C: The liquid is slowly flowing into the diver. The volume of the liquid sucked may be calculated by the difference between the volume of the liquid previously introduced and that of the liquid left in the capillary after the operation.

D: The solution contained in the neck of the diver is mixed with the inhibitor by means of some blowing and sucking of a part of it in a drop of paraffin oil.

E: The micropipette is closed with the microforge under microscope control.

1. Dimensions of the diver.

The small volume of material (0.1—0.01 μl) utilized in this investigation equired a special typer of diver in order to obtain adequate sensitivity for ChE determinations. The modified diver is shown schematically in Fig. 1 A and B and has the following dimensions: Inside neck diameter = 10 μ, neck length = 2.5 mm, neck wall thickness = 3 μ, inside tail diameter = 10 μ, tail wall thickness = 3 μ, tail length = 6 mm, tip diameter = 1 μ, bulb diameter = 200 μ, bulb volume = 0.0042 μl, total volume of the diver = 0.0049 μl, total weight = 45 μg.

2. Preparation of the diver.

All diver micropipettes employed in the present investigation were entirely made by means of the simple microforge shown in Fig. 4 C and D, which is supplied with an electrically heated platinum wire (diam. = 0.15 mm) the temperature of which may be regulated by means of a variable resistance in the circuit.

All operations are done under microscope control.

The preparation procedure of the diver micropipettes consists of three steps: starting from glass tubes (Phoenix "workable heat resisting glass") having an inside diameter of 8.5 mm and a thickness of about 0.9 mm, smaller and thin walled tubes (inside diameter about 1 mm and thickness 0.05 mm) are drawn. From these thin tubes capillaries having an inside diameter of about 20—10 μ and a thickness of 5—3 μ are then drawn out in the microflame.

These capillaries are the real starting material of the diver micropipettes. A 1—2 cm long segment of the capillary is connected to the holder of the microforge as shown in Fig. 4 C and D and by means of two successive operations a spherical bulb is made. A tip of 1 μ or less was pulled at one extremity according to the technique described by DE FONBRUNE (1949).

A picture of a diver prepared according to this procedure is shown in Fig. 1 A and B.

3. Chargement of the diver.

The diver micropipette was employed in this investigation as a micropipette in sucking up the cellular or nuclear material (see Fig. 3 F). The substrate bicarbonate solution and a small quantity of 5 % CO_2/95 % N_2 was first introduced into the diver according to the technique described by ZAJICEK and ZEUTHEN (1956).

The substrate used was 5.3×10^{-4} M solution of ACh (acetylcholine iodide Hoffmann La Roche). The incubation medium (pH ca 7.4) was generally prepared by dissolving 14.5 mg ACh in 10 ml of a solution made by adding 9.6 ml 0.85 % NaCl to 10 ml 0.199 % $NaHCO_3$ and 0.4 ml 1.76 % $CaCl_2 \cdot 6\,H_2O$, and saturated with a 5 % CO_2/95 % N_2 gas mixture.

All the filling operations had to be done under microscopic control. For this purpose a common dissection microscope was used at a magnification of about 80 ×.

The following four different preparations were used:

1) isolated anterior horn cells for aspiration of material both from cytoplasm and nucleus;

2) single nuclei liberated from the cytoplasmic substance by means of micromanipulation;

3) occasionally isolated nuclei found in the spinal cord suspension;

4) isolated nucleoli.

In Fig. 3 A and B single isolated nuclei are shown at the same magnification.

Fig. 3 F illustrates the aspiration procedure of nuclear material from single isolated nuclei.

After the diver had been charged with the buffer-substrate solution and the cellular material, it was rapidly closed at one end by means of the microforge and then placed in the floatation vessel (Fig. 2 E). By means of this closing procedure, the diffusion of the gas through the neck could be prevented.

4. Introduction of the inhibitor in the diver.

In order to differentiate between AChE and ChE a selective inhibitor, Mipafox (bis-monoisopropylaminophosphoryl fluride) was used. As has recently been demonstrated by ALDRIDGE (1953), DAVISON (1953) and HOLMSTEDT (1957), this substance completely inhibits the non spec. ChE from human serum at a concentration of 8×10^{-7} M whereas it does not affect the AChE of human erythrocytes at a concentration lower than 6×10^{-6} M.

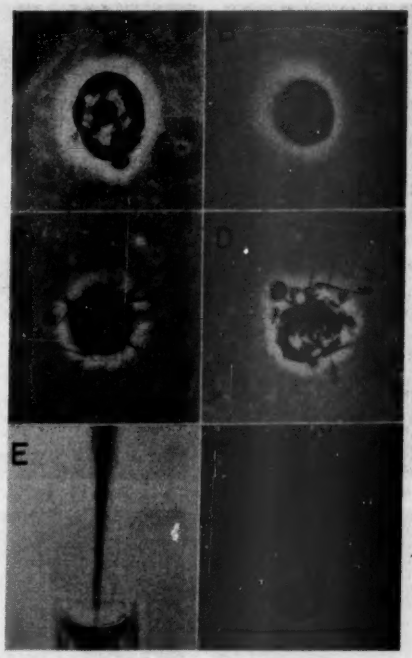

Fig. 3.

A and B: show two single nucleus preparations of anterior horn cells of the spinal cord of rat isolated by means of micromanipulation. The nucleus in B showed an activity of 2.6×10^{-6} µl CO_2/hour.

C and D: show the ChE activity demonstrated by thin needle shaped Cu-thiocholine sulfate crystals all over and inside the isolated nucleus preparations. Thiocholine histoch. method. Incubation time: about 180 min. Magnification in pictures A, B, C and D about 800 ×.

E: Filling the diver with the inhibitory substance. The tip of the diver (diam. about 1 µ) is introduced in a capillary (diam. about 10 µ) containing a small quantity of inhibitor. In the picture the tip is already filled with the substance.

F: The tip of the micropipette is shown inside a nucleus of an anterior horn cell of rat while the nucleoplasm is sucked in. The operations E and F are done in a drop of paraffin oil. (See text.)

The controls which have been performed by GIACOBINI and HOLMSTEDT (1958), on the ChE of rat brain and spinal cord confirmed that a 5×10^{-6} M solution of Mipafox did not affect the AChE while non spec. ChE activity was completely inhibited.

The quantitative determination of ChE and AChE per nerve cell or

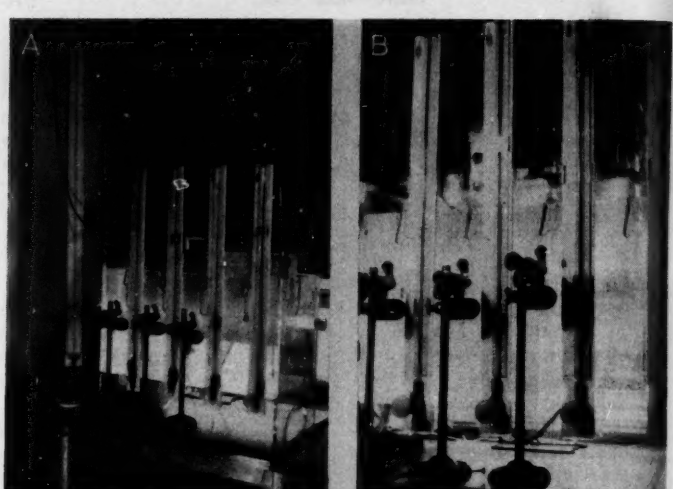

Fig. 4.

A: Cartesian diver apparatus, assembly.
B: Detail picture showing the 4 micromanometers.
C: The simple microforge described in the text.
D: Detail picture of the apparatus during the preparation of the bulb and the tip of the diver.

cell part was carried out by the following procedure previously reported by GIACOBINI and ZAJICEK (1956).

a) determination of the total cholinesterase activity by the hydrolysis of ACh;

b) determination of the AChE activity alone after inhibition of the non spec. ChE by the introduction into the diver of known quantity of a 5×10^{-6} M solution of Mipafox.

Control experiments were carried out with a known quantity of human serum ChE.

The procedure for the introduction of the inhibitor in the diver is shown schematically in Fig. 2. According to this technique a known quantity of the inhibitor can be introduced in the diver with an error of about 10 %. As demonstrated by the inhibition curves for rat brain (DAVISON 1953, GIACOBINI and HOLMSTEDT 1958) an error in this order of magnitude does not significantly interfere with the estimation of the AChE activity.

AUGUSTINSSON and NACHMANSOHN (1949) studying the kinetics of the inhibition of ChE observed a protective action if ACh was added to the enzyme simultaneously with the inhibitor. This protective action was probably due to a competitive action on the same active centre of the enzyme. Higher concentrations of the inhibitor are therefore required in order to obtain the same degree of inhibition. In the above described technique, 10—20 times higher concentrations of the inhibitor can be used. This proved to be high enough to produce the total inhibition of non spec. ChE without affecting AChE (DAVISON 1953, GIACOBINI and ZAJICEK 1956, GIACOBINI and HOLMSTEDT 1958).

5. Type of substrate.

In all the experiments reported ACh was used as substrate. AUGUSTINSSON and ISACHSEN (1957) found that when thiocholine esters are employed in esterase determinations, the total amount of CO_2 produced is higher than expected from a theoretical point of view; this, probably, is due to the acidic property of thiocholine at the pH of the reaction mixture, as also demonstrated by HANSEN (pers. comm.) in non-enzymatic experiments. Therefore, when calculating the equivalent amount of substrate hydrolysed (acetylthiocholine) a correction for the values obtained by means of the diver technique must be considered.

A series of substrates, BuCh (butyrylcholine), MeCh (acetyl-β-methylcholine) and BzCh (benzoylcholine) together with two specific inhibitors (Mipafox and BW 284 C 51) have been employed in experiments on different segments of single nerve fibers innervating various muscle structures. The results of this investigation will be published elsewhere (GIACOBINI 1959 b).

6. Sensitivity of the method.

In order to check the theoretically calculated sensitivity and accuracy of the diver, control experiments were carried out with a known quantity of human serum ChE and with samples of the Ringer bicarbonate-ACh solution. In the latter case, after an initial period of about 30—45 min,

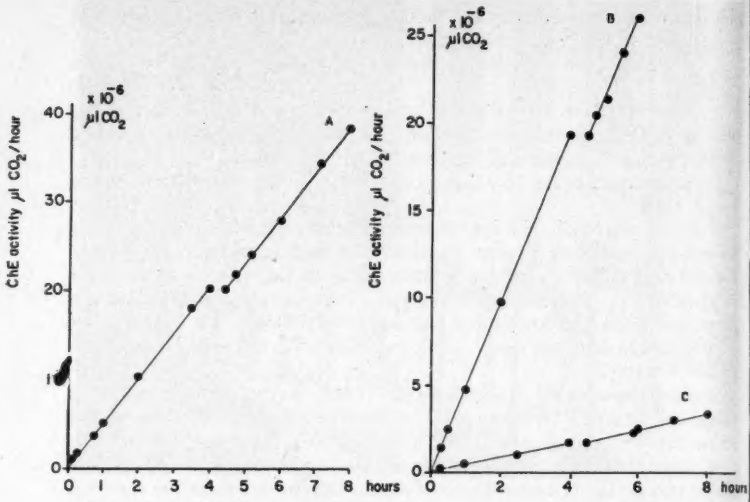

Fig. 5.

A: ChE activity curve of an isolated nucleus of a large anterior horn cell (diam. 11 × 5 µ, volume 0.086μ^3 × 10^3).

B: ChE activity curve of a sample of cytoplasmic material of about 0.01 $\mu\mu$l.

C: ChE activity curve of a sample of nucleoplasmic material of about 0.01 $\mu\mu$l.

the readings on the scale were constant within 15—25 mm scale (3.5—7.5 mm H_2O) over a period of 12 h.

The sensitivity of the method could be calculated to be about 1×10^{-6} µl/CO_2/hour with a ± 15 % accuracy.

In a previous work (GIACOBINI and ZAJICEK 1956) it has been claimed a sensitivity of 2×10^{-6} µl CO_2 per hour, which is an overestimation; in fact the real sensitivity of that method was about 1×10^{-4} µl with ± 5 % accuracy. In the present method this sensitivity could be increased about 100 times by reducing about 100 times the dimension of the diver (V_g and V_f in the formula at page 316).

Results.

Typical ChE activity curves.

Some typical ChE curves obtained by means of the described technique are shown in Fig. 5. Fig. 5 A shows the ChE activity

curve of an isolated nucleus of a large anterior horn cell having the max. and min. diam. of 11 μ and 5 μ, and a volume of 0.086 $\mu^3 \times 10^3$. The total AChE activity of this nucleus was estimated to be 5.1×10^{-6} μl CO_2/hour corresponding to the hydrolysis of about 660 pg ACh (1 pg = 10^{-12} g).

The intervals of the curve indicate where the experiment has been interrupted for the addition of the inhibitor.

Fig. 5 B demonstrates the ChE activity curve of a sample of cytoplasmic material of about 0.001 $\mu\mu$l: the ChE activity was 4.8×10^{-6} μl CO_2/hour corresponding to 625 pg ACh.

Finally a 0.01 $\mu\mu$l sample of nucleoplasmic material is shown in Fig. 5 C; as seen from the curve this showed an activity of about 0.41×10^{-6} μl CO_2/hour corresponding to 53 pg ACh.

Discussion and Conclusion.

The necessity of taking very cautiously the results on the localization of enzymes in the various fractions obtained with homogenates of tissues has been stressed by several authors (ALLFREY, MIRSKY and STERN 1955, BRADFIELD 1950, GLICK 1953, HOLTER 1952, HOLTER and LINDERSTRÖM-LANG 1951, KOPAC 1950, 1955, NOVIKOFF 1956, NOVIKOFF *et al.* 1953, SCHNEIDER and HOGEBOOM 1956).

The biochemistry of cellular particles had been recently critically reviewed by SCHNEIDER and HOGEBOOM (1956). In this article the authors emphasize the necessity of checking the results obtained by cell fractionation methods with other independent methods.

ALLFREY, MIRSKY and STERN in another recent review on the chemistry of the nucleus (1955) undertake a critical examination of the procedures generally used for the study of enzymes of the nucleus. These authors indicate the necessity of new methods for the isolation of intact nuclei. According to these authors two principal conditions have to be satisfied for this purpose; elimination of the contamination of the isolated nuclei with particles of other origin and avoidance of alteration of the nuclear membrane due to mechanical treatment and to the composition of the homogenizing medium.

The heterogenity of the fractions themselves and the difficulty of identifying the isolated homogenate particulates from particles

present in intact cells has also been discussed earlier (KOPAC 1955, NOVIKOFF 1956, NOVIKOFF *et al.* 1953).

The passage of the enzyme molecules through the cell- and nuclear-membrane with resulting distorsion of the localization and the stability of the enzyme are two factors which have to be taken into consideration when employing techniques involving manipulation and preservation of the tissue. When cells or nuclei are isolated by careful dissection as, in the case of the experiments reported in this paper, several factors make it unlikely that AChE could diffuse both inside and outside the cellular structures; first the big size of the enzyme molecules (m. w. about 3,000,000 for the electric organ AChE (ROTHENBERG and NACHMANSOHN 1947), second, the abundant evidence indicating that the enzyme is firmly bound to cytoplasmic structures both in nerve cells (NATHAN and APRISON 1955, TOSCHI 1959), erythrocytes (AUGUSTINSSON 1948, BRAUER and BOOT 1945, PALEUS 1957) and platelets (ZAJICEK 1957).

In order to study the possibility of diffusion of the enzyme from the cell to the medium in which the cell was placed, the ChE activity of the medium was tested both histochemically and with the cartesian diver method. It was seen that such diffusion does not happen even when cell has been kept some hours in the incubation (GIACOBINI 1956, 1957 a).

In the case of histological preparations, HOLT (1954) found that washing tissue sections for several hours does not reduce the ChE activity of the tissue. Furthermore, storage in aqueous solution of formaline fixed muscle sections for as long as one month had no effect on the localization of the enzyme by histochemical staining.

Regarding the question of the stability of the enzyme molecule it has been known since the early studies of GLICK (1937) that AChE is a remarkably stable enzyme: no significant change of activity was found in nuclei or brain tissue suspensions kept at 1°—4° for periods up to 20 days (RICHTER and HULLIN 1951). Similarly, it was found that a 42 years old sample of horse blood preserved at room temperature contained up to 85 % of its original ChE activity (KEILIN and WANG 1947).

In conclusion it can be said that the principal difficulty encountered when studying the intracellular localization of ChE in nerve cells is the possibility of contamination with material of different origin. For this reason neither the demonstration with

histochemical methods on single cells (GIACOBINI 1956) nor the determination with the diver technique of the activity of isolated cells or single nuclei in toto can be taken as definitive evidence of the cytoplasmic or nuclear localization of the enzyme. Only the demonstration of the enzyme in samples of nucleo- and cytoplasm may be a sure indication. Therefore, on this basis a complete study of the intracellular localization of ChE in nerve cells was undertaken (GIACOBINI 1959 b) using the technique described in this paper.

The procedure reported in this article, and the results obtained with it, suggests the possibility of studying the enzyme activity on parts of cells isolated from relatively intact cell preparations. The same technique may also prove useful in the future for the study of the intracellular distribution of other enzymes, the determination of which lend themselves to gasometrical techniques (*e. g.* other esterases, lipolytic enzymes, proteolytic enzymes, cocarboxylase etc.) or for the study of the respiration of different cellular structures.

Summary.

1. The procedure for the determination of ChE activity in $\mu\mu$l samples of intracellular material is described.

The method is an application of the cartesian diver technique of LINDERSTRÖM-LANG which allows quantitative determinations in the order of 1×10^{-6} μl CO_2/hour with $\pm$ 15 % accuracy.

2. The method has been employed in determinations of ChE activity in small samples of cytoplasm and nucleoplasm removed by means of a diver micropipette technique from anterior horn cells of the spinal cord of rat.

3. A sample of 0.01 $\mu\mu$l cytoplasm was found to exhibit a ChE activity of 4.8×10^{-6} μl CO_2/hour corresponding to the hydrolysis of about 625 pg acetylcholine and a sample of 0.01 $\mu\mu$l nucleoplasm showed a ChE activity of 0.41×10^{-6} μl CO_2/hour corresponding to the hydrolysis of about 55 pg acetylcholine.

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References.

ALDRIDGE, W. N., Biochem. J. 1953. *53*. 62.

ALLFREY, V. G., A. E. MIRSKY and H. STERN, Advanc. Enzymol. 1955. *16*. 411.

AMMON, R., Pflügers Arch. ges. Physiol. 1933. *233*. 486.

AUGUSTINSSON, K. B., Acta physiol. scand. 1948. *15*. Suppl. 52.

AUGUSTINSSON, K. B. and D. NACHMANSOHN, J. biol. Chem. 1949. *179*. 543.

AUGUSTINSSON, K. B. and THORHILD ISACHSEN, Acta Chem. scand. 1957. *11*. 750.

BENSLEY, R. R. and N. L. HOERR, Ant. Rec. 1934. *60*. 251.

BRADFIELD, I. R. G., Biol. Rev. 1950. *25*. 13.

BRAUER, R. W. and M. A. BOOT, Fed. Proc. 1945. *4*. 113.

CLAUDE, A. J., J. exp. Med. 1946. *84*. 51.

DAVISON, A. N., Biochem. J. 1953. *54*. 583.

DE FONBRUNE, P., Technique de micromanipulation — Mon. Inst. Pasteur. (Paris) 1949.

GIACOBINI, E., Acta physiol. scand. 1956. *36*. 276.

GIACOBINI, E., J. Neurochem. 1957 a. *1*. 234.

GIACOBINI, E., Acta physiol. scand. 1957 b. *42*. Suppl. 145. 49.

GIACOBINI, E., Acta physiol. scand. 1959 a. *45*. 238.

GIACOBINI, E., Acta physiol. scand. 1959 b. *45*. Suppl. 156.

GIACOBINI, E. and J. ZAJICEK, Nature (Lond.) 1956. *177*. 185.

GIACOBINI, E. and B. HOLMSTEDT, Acta physiol. scand. 1958. *42*. 12.

GLICK, D., Biochem. J. 1937. *31*. 521.

GLICK, D., Intern. Rev. Cytol. 1953. *2*. 447.

HANSEN, B., To be published. Personal communication.

HOGEBOOM, G. H., W. C. SCHNEIDER and G. E. PALADE, Proc. Soc. exp. Biol. (N. Y.) 1947. *65*. 320.

HOGEBOOM, G. H., W. C. SCHNEIDER and G. E. PALADE, J. biol. Chem. 1948. *172*. 619.

HOLMSTEDT, B., Acta physiol. scand. 1957. *40*. 322.

HOLT, S. J., Proc. roy. Soc. B 1954. *142*. 160.

HOLTER, H., Advanc. Enzymol. 1952. *13*. 1.

HOLTER, H. and K. LINDERSTRÖM-LANG, Physiol. Rev. 1951. *31*. 432.

KEILIN, D. and Y. L. WANG, Biochem. J. 1947. *41*. 419.

KOELLE, G. B. and Y. S. FRIEDENWALD, Proc. Soc. exp. Biol. (N. Y.) 1949. *70*. 617.

KOPAC, M. J., Ann. Revs. Physiol. 1950. *12*. 7.

KOPAC, M. J., Int. Rev. Cytol. 1955. *IV*. 1.

KOPAC, M. J. and J. HARRIS, Anat. Rec. 1951. *111*. 116.

LINDERSTRÖM-LANG, K. Nature (Lond.) 1937. *140*. 108.

LINDERSTRÖM-LANG, K. and D. GLICK, C. R. Lab. Carlsberg. Ser. Chim. 1938. *22*. 300.

MICKLEWRIGHT, H. L., N. B. KURNICK and R. HODES, Exp. Cell. Res. 1953. *4*. 151.

NATHAN, P. and M. H. APRISON, Fed. Proc. 1955. *14*. 106.

NOVIKOFF, A. B., Science 1956. *124*. 969.
NOVIKOFF, A. B., E. PODBER, J. RYAN and E. NOE, J. Histoch. Cytoch. 1953. *1*. 27.
PALEUS, S., Arch. Biochem. 1957. *12*. 153.
RICHTER, D. and R. P. HULLIN, Biochem. J. 1951. *48*. 406.
ROTHENBERG, M. A. and D. NACHMANSOHN, J. Biol. 1947. *168*. 223.
SCHNEIDER, W. C. and G. H. HOGEBOOM, Cancer Res. 1951. *11*. 1.
SCHNEIDER, W. C. and G. H. HOGEBOOM, Ann. Rev. Biochem. 1956. *25*. 201.
TOSCHI, G., Exp. Cell. Res. 1959. In press.
ZAJICEK, J., Acta physiol. scand. 1957. *40*. Suppl. 138.
ZAJICEK, J. and E. ZEUTHEN, Exp. Cell. Res. 1956. *11*. 568.
ZEUTHEN, E., Biol. Bull. 1950. *98*. 139.
ZEUTHEN, E., J. Embryol. exp. Morph. 1953. *1*. 239.
ZEUTHEN, E., Biol. Bull. 1955. *108*. 366.

Acta physiol. scand. 1959. 45. 328—338.

From the Department of Histology, University of Lund, Lund, Sweden.

Nucleotides in the Catechol Amine Granules of the Adrenal Medulla.

By

N.-Å. HILLARP and G. THIEME.

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Abstract.

HILLARP, N.-Å. and G. THIEME. Nucleotides in the catechol amine granules of the adrenal medulla. *Acta physiol. scand.* 1959. *45.* 328—338. — The work was carried out to determine whether the catechol amines in the adrenal medulla from birds and mammals are stored together with adenosinephosphates in the same way as previously found in the cow. The amine granules of the adrenal medulla from fowl, goose, cat and goat were isolated by means of density gradient centrifugation. As in the cow no nucleotides other than adenosinephosphates were found (ion exchange and paper chromatography, high voltage paper electrophoresis). No purine or pyrimidine bases or nucleotides were demonstrable. In all animals examined ATP was predominant, but ADP and AMP were always present. The relative proportions of the three phosphates varied rather markedly, however. In spite of this, amines and adenosinephosphates were regularly present in practically equivalent amounts.

In the cow adrenal medulla the specific granules storing the catechol amines (adrenaline and noradrenaline) contain adenosinephosphates in amounts practically equivalent to the amine content (HILLARP, HÖGBERG and NILSON 1955, FALCK, HILLARP and HÖGBERG 1956, BLASCHKO *et al.* 1956, HILLARP 1958 b). The

assumption that the adenosinephosphates play an important role in the storage and release of the amines has been supported by other observations (CARLSSON and HILLARP 1956, CARLSSON, HILLARP and HÖKFELT 1957, SCHÜMANN 1958, CARLSSON and HILLARP 1958, HILLARP 1958 d).

In granules from cow adrenal medulla the adenosinephosphates are made up mainly (87 per cent) of ATP and only small amount of ADP (10 per cent) and AMP (3 per cent) are present (HILLARP 1958 b). The experiments reported below were carried out to determine whether the catechol amines are stored together with adenosinephosphates in the same way in other animals belonging to two different classes (birds and mammals).

Methods.

Hens (common domestic fowls) (body weight, 2 to 2.5 kg) and male geese (*Anser anser*) (5 to 6 kg) were killed by decapitation. Goats (one male, 35 kg, and two females, 35 and 15 kg) were killed by a blow on the head and subsequently bled. Cats (four males and two females, 3 to 3.7 kg) were rapidly made unconscious with carbon monoxide and then decapitated. The adrenals were removed without delay and chilled with ice. The medulla was dissected free from cortical tissue (the entire glands of the birds were used), cut into small pieces in 0.3 M sucrose and homogenized (HILLARP, LAGERSTEDT and NILSON 1953). The samples were kept at 0° C throughout the preparation.

Cell fractionation: The homogenate was freed from cells, nuclei, connective tissue, etc., by centrifugation at 800 × *g* for 6 min. The amine granules of the supernatant were spun down (together with mitochondria and some microsomal material) at 23,000 × *g* for 20 min (MSE superspeed 20' refrigerated centrifuge; angle head 8 × 10 ml) and then resuspended in 0.3 M sucrose. The suspension was distributed to nylon tubes (fitting to the swing-out head of the MSE centrifuge) containing a density gradient (0.5 ml of 1.8 M, 0.5 ml of 1.5 M and 1.0 ml of 1.2 M sucrose) which had been kept at 0° for 5 hours. After 30 min the tubes were centrifuged at 50,000 × *g* for 2 hours. In this way a sediment of amine granules is obtained practically free from contaminating structures (HILLARP 1958 c). The supernatant was sucked off and the inside of the tubes carefully cleaned. The sediments (G1) were extracted with 0.4 N perchloric acid (PCA) or with 70 per cent ethanol (— 10°) and the proteins were immediately spun down. The protein residue was washed once. The PCA extracts were neutralized with chilled potassium hydroxide and kept at — 4° overnight to precipitate the potassium perchlorate. The ethanol extracts were kept at — 25° until the following day, when they were analyzed.

Some of the amine granules did not sediment under the conditions

used but were suspended immediately above the bottom granules. In some experiments (Table I) this fraction (G2), which consisted mainly of adrenaline containing granules, was sucked off and collected in a volume of PCA or ethanol to give a final concentration of 0.4 N and 70 per cent respectively. The extracts were treated in the way described above.

Unless otherwise stated, all operations were performed without any delay and at 0° in order to minimize any changes in the granules. Control experiments showed no ATP break-down during the extraction and assay procedures (cf. HILLARP 1958 b).

All fractions obtained during the cell fractionations were analyzed for catechol amines. This showed that the degree of homogenization was more than 80 per cent for the birds and over 90 per cent for the mammals and that more than 85 per cent of the amines in the cell-free homogenate were localized to the specific granules.

Assay methods: Inorganic (P_o) and acid-labile phosphate (P_s; hydrolysis at + 100° in N HCl for 8 min) was determined according to FISKE and SUBBAROW (LEPAGE 1951), adrenaline and noradrenaline according to VON EULER and HAMBERG (1949) and protein-N by the biuret method (CLELAND and SLATER 1953). Duplicate determinations were made of P and amines.

In the calculation of the total catechol amine content of G1 and G2, a value used for the molecular weight was computed from the percentage of adrenaline and noradrenaline in the extracts.

Ion exchange chromatography: An aliquot of the extract was passed through a cation exchange column (Dowex 50, 150 to 300 mesh, 20 × 8 mm, pH 6) which permits AMP to pass through quantitatively. The effluent was brought to pH 9 with ammonium hydroxide and passed through an anion exchange column (Dowex 2, 200 to 400 mesh, 23 × 6 mm, Cl^-). The nucleotides were eluted in 10 or 3 ml fractions (at the rate of 0.2 ml/min) according to COHN (1951). The ultraviolet absorption of the various fractions was read in a Beckman spectrophotometer, model DU, with the eluants as blanks. Complete ultraviolet spectra of the fractions were taken at pH 2, 7 and 11. The nucleotides were then adsorbed on Norit (CRANE and LIPMAN 1953) and eluted with 50 per cent ethanol containing 2 per cent ammonium hydroxide. The eluates were concentrated *in vacuo* and examined by paper electrophoresis. The remaining part was hydrolyzed in N HCl at + 100° for one hour, concentrated again and examined by paper chromatography.

The catechol amines retained by the cation exchanger were eluted with N HCl and the amine content was determined both colorimetrically and by ultraviolet absorption.

Paper electrophoresis: An aliquot of the original ethanol extracts was evaporated in a stream of N_2 and the residue was dissolved in a small volume of water. The components were separated by high voltage electrophoresis on Whatman no. 1 paper immersed in carbon tetrachloride at about + 10° (acetic acid-acetate buffer of ionic strength 0.1 and pH 4.15, 3 kV for 40 min, 55 V/cm). The ultraviolet absorbing substances were located by making a contact print by ultraviolet light (Philips TUV,

15 W lamp). The spots and suitable blanks were cut out and eluted with 1.0 ml of 0.1 N HCl and the ultraviolet spectra of the eluates were taken. Recovery experiments showed that ATP, ADP and AMP could be separated and quantitatively determined in amounts down to about 3 μg.

Paper chromatography: The hydrolized nucleotides (see above) were subjected to ascending single dimensional chromatography on Whatman no. 1 paper in two different solvent systems: isopropanol — NH_3 (HERSHEY, DIXON and CHASE 1953) and isopropanol — HCl (WYATT 1951). The spots were located and eluted in the way described above.

Reference substances: The same substances and the same molecular extinction coefficients were used as in a previous study (HILLARP 1958 b).

Results.

Catechol amines.

The amine granules from cow adrenal medulla isolated by density gradient centrifugation in a similar way as in the present work show no or practically no contamination with mitochondria or microsomes (HILLARP 1958 c). They have a very high and rather constant content of catechol amines (about 35 μmoles/mg protein-N). Although the animals studied by us belong to two different classes, they were not found to differ appreciably in this respect from the cow (Table I). Some of the values approached but never exceeded the highest value observed in the cow (37 μmoles/mg N). This upper limit may represent the maximum storing capacity of the granules.

The amine granules (G2) of lower density (or lower sedimentation rate) have — as found for the cow (HILLARP 1958 c) — an appreciably lower amine content. They contain almost exclusively adrenaline in accordance with observations in other studies (EADE 1956, SCHÜMANN 1957, HILLARP 1958 c).

Nucleotides and related substances.

The nucleotides found in the granules were identified as adenosinephosphates by the following criteria. 1) They showed the same elution positions on anion exchange chromatography and the same electrophoretic mobility as AMP, ADP and ATP (Fig. 1 to 3). 2) The ultraviolet absorbing substances eluted on anion exchange chromatography and after electrophoresis had the same ultraviolet spectra at pH 2, 7 and 11 as pure adenosinephosphates. 3) After hydrolysis paper chromatography revealed only one ultra-

Table I.

Content of catechol amines and adenosinephosphates in medullary granules isolated by density gradient centrifugation.

Animals	Number of Animals	Granule Fraction	Extraction	Catechol Amines			Adenosinephosphates per μmole Amines			
				Total μmoles	μmoles per mg N	Noradr. Per cent	ATP μmoles	ADP μmoles	AMP μmoles	Total μequival
Fowl 1	8	G1	PCA	17.5	29.2	51	0.173 (60)	0.092 (32)	0.023 (8)	1.01
Fowl 2	10	G1	PCA	24.6	35.1	42	0.203 (69)	0.068 (23)	0.024 (8)	1.06
Fowl 3	10	G1	Ethanol	30.2	33.9	31	0.202 (66)	0.073 (24)	0.029 (10)	1.08
Goose	2	G1	Ethanol	8.00	34.8	60	0.198 (76)	0.043 (17)	0.018 (7)	0.96
Cat	6	G1	Ethanol	6.28	28.2	58	0.187 (69)	0.061 (22)	0.023 (9)	0.98
Goat 1	1	G1	PCA	5.33	35.6	36	0.199 (78)	0.048 (19)	0.009 (4)	0.96
Goat 2	1	G1+G2	Ethanol	11.3	—	32	0.208 (79)	0.046 (17)	0.011 (4)	0.99
Goat 3	1	G1+G2	Ethanol	6.52	—	34	0.227 (81)	0.037 (13)	0.015 (5)	1.05
Cow	—	G1	—	—	34.7	—	0.221 (87)	0.026 (10)	0.008 (3)	0.98
Fowl 1	8	G2	PCA	13.0	22.1	13	0.158 (55)	0.098 (34)	0.030 (10)	0.99
Fowl 2	10	G2	PCA	6.45	21.0	2	0.169 (58)	0.085 (29)	0.039 (13)	1.01
Fowl 3	10	G2	Ethanol	5.20	19.4	3	0.188 (66)	0.072 (25)	0.026 (9)	1.02
Goat 1	1	G2	PCA	5.70	19.8	9	0.200 (80)	0.042 (17)	0.009 (3)	0.94

G1, the granules of the bottom sediment; G2, the granules suspended above this sediment. Goat 3 was a kid. The values for cow granules were obtained in previous works (HILLARP 1958 b and c).

The figures in parentheses are per cent of total adenosinephosphates.

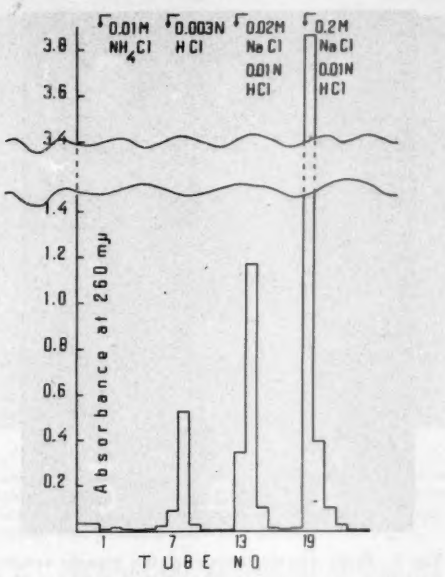

Fig. 1. Anion exchange chromatography (according to COHN 1951) of half of the granule extract (G1) from fowl 3 (Table I). Before chromatography the catechol amines were removed by a cation exchanger.

violet absorbing substance, which showed the same position as adenine in both solvent systems used and had the same spectrum. 4) Observed and calculated P_s values for the ADP and ATP fractions obtained by anion exchange chromatography were in good agreement.

No nucleosides, bases or nucleotides other than the adenosinephosphates were detected. Minor amounts of such compounds especially of those with a comparatively weak absorbancy at 250 to 260 mμ cannot be excluded. However, when extracts had passed through the cation exchanger they showed an almost pure adenine spectrum. The ultraviolet absorption spectrum of the substances taken up by the cation exchanger and eluted with HCl was practically identical with that of adrenaline and calculations of the catechol amine content by this method were in good agreement with the colorimetrical determinations.

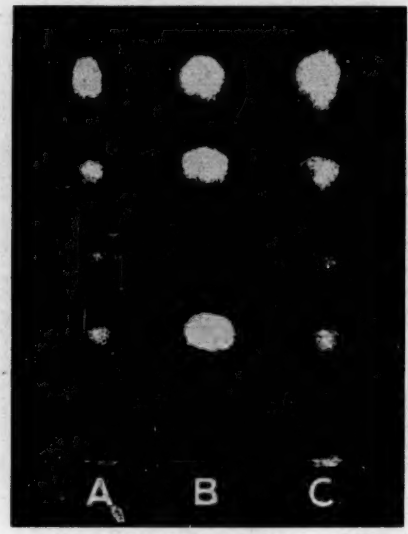

Fig. 2. Paper electrophoresis of the granule extract (G1) from cat (Table I). The separation was carried out on Whatman no. 1 paper using NaAc-HAc buffer, pH 4.15, 55 V/cm for 40 min at about + 10°.

A: 10 µl of the extract;

B: standard containing ATP (top spot), ADP and AMP (about 20 µg of each);

C: 20 µl of the extract. Contact print by ultraviolet light.

After electrophoresis of the extract from cat, however, a very small spot appeared halfway between the AMP and ADP spots (Fig. 2). It has as yet not been possible to identify this substance.

The adenosinephosphate content of the amine granules, as judged by electrophoresis (mean value from two analyses), was in close agreement with the values found on anion exchange chromatography.

In all animals examined ATP was the dominating nucleotide, but ADP as well as AMP were always present (Table I). Their relative proportions, calculated as a percentage of the total adenosinephosphates, varied considerably, however, the cow having the lowest and the fowls the highest percentage of ADP and AMP.

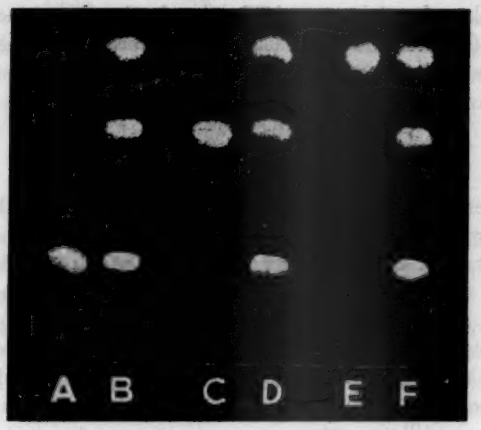

Fig. 3. Paper electrophoresis of the three fractions obtained at anion exchange chromatography of the granule extract from fowl 3 (see Fig. 1). The nucleotides were adsorbed on Norit, eluted and concentrated. The separation and printing were carried out as in Fig. 2.

A, C and E: the AMP, ADP and ATP fractions respectively;

B, D and F: standard containing ATP, ADP and AMP (10 μg of each).

In spite of this the total adenosinephosphates represented an amount of acid practically equivalent to the amines in the granules (Table I, last column). The ATP values for the fowls are in good agreement with the values found by SCHÜMANN (1958) using the firefly method.

Practically speaking, the extracts contained no acid-labile phosphates other than ATP and ADP, the adenosinephosphates representing 94 to 96 per cent of the total P_s. The inorganic phosphate content was low (0.02 to 0.03 μmoles/μmole amines).

The fraction (G2) containing almost pure adrenaline granules was analyzed in some of the experiments (Table I). The somewhat low ATP content found in the first two experiments was probably due to a technical error. The tubes in which the granules were transferred after the centrifugation did not contain PCA or ethanol. Consequently some of the granules may have been damaged by osmotic lysis (cf. HILLARP and NILSON 1954) and the liberated

ATP broken down by the ATPase and adenylate kinase of the granules (HILLARP 1958 a). There is thus probably no significant difference between adrenaline and noradrenaline granules in respect to nucleotides.

Discussion.

In several respects the amine granules isolated from the adrenal medulla of birds and of mammals are similar. They have the same high content of amines and nucleotides per mg protein-N. At most traces of nucleotides other than ATP, ADP and AMP are present and the adenosinephosphates and amines occur in practically equivalent amounts in the granules. There is one difference, however, in that the relative proportions of the three phosphates vary rather markedly from animal to animal. The significance of this difference is still obscure.

It might be argued that the ADP and AMP found in the granules are merely split products from ATP which may have broken down before the extraction. Some arguments put forward in a previous work (HILLARP 1958 b) militate against this possibility. In addition, the present findings strengthen one of those arguments. Since the ATP in the isolated granules is stable at 0° (FALCK, HILLARP and HÖGBERG 1956, HILLARP 1958 b) the postulated break-down probably must have occurred before the adrenals could be chilled. Since the glands could hardly ever be removed from the cow within less than half an hour of death, one would expect large amounts of ADP and AMP. But of all animals examined, the lowest values were found for the cow and the highest for the fowls, from which the adrenals were removed and cooled within a few minutes of death of the birds.

Despite the differences between the proportions of ATP, ADP and AMP in the granules isolated from the various animals studied, the total adenosinephosphates represented an amount of acid practically equivalent to the catechol amines (Table I, last column). This supports the view (HILLARP 1958 b) that the amines are stored in intimate connection to the adenosinephosphates. The osmotic properties of the granules and the amine concentration found in them make it necessary to assume that the amines and nucleotides are incorporated in a complex molecular structure preventing them from forming free ions or molecules in the intragranular water (CARLSSON and HILLARP 1958). The presence of large amounts

(77 per cent of the total granule proteins) of a special protein fraction that is released together with the amines and nucleotides on osmotic lysis of the granules (HILLARP 1958 c) suggests that the intragranular proteins may be an essential part of the postulated structure.

Summary.

1. The catechol amine granules of the adrenal medulla from fowl, goose, cat and goat were isolated by means of density gradient centrifugation.

2. The granules were found to have the same high content of amines as those from cow medulla. The values found suggest a maximum storing capacity of about 35 to 37 μmoles/mg protein-N.

3. As in the cow no nucleotides other than adenosinephosphates were found. In all animals examined ATP was predominant, but ADP and AMP were always present. The relative proportions of the three phosphates varied rather markedly, however. In spite of this, amines and adenosinephosphates were regularly present in practically equivalent amounts. This further supports the view that the amines are stored in intimate connection to the adenosinephosphates.

4. No purine or pyrimidine bases or nucleosides were demonstrable in the granules. The inorganic phosphate content was low (0.02 to 0.03 μmoles/μmole amines).

5. There is probably no significant difference between adrenaline and noradrenaline granules in respect to their nucleotide content.

We thank Dr. R. BERGKVIST, Department of Biochemistry, for valuable advice concerning paper electrophoresis and chromatography.

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References.

BLASCHKO, H., G. V. R. BORN, A. D'IORIO and N. R. EADE, Observations on the distribution of catechol amines and adenosinetriphosphate in the bovine adrenal medulla. *J. Physiol.* (Lond.) 1956. *133*. 548—557.

CARLSSON, A. and N.-Å. HILLARP, Release of adenosine triphosphate along with adrenaline and noradrenaline following stimulation of the adrenal medulla. *Acta physiol. scand.* 1956. *37*. 235—239.

CARLSSON, A. and N.-Å. HILLARP, On the state of the catechol amines of the adrenal medullary granules. *Acta physiol. scand.* 1958 *44*. 163—169.

CARLSSON, A., N.-Å. HILLARP and B. HÖKFELT, The concomitant release of adenosinetriphosphate and catechol amines from the adrenal medulla. *J. biol. Chem* 1957. *227*. 243—252.

CLELAND, K. W. and E. C. SLATER, Respiratory granules of heart muscle. *Biochem. J.* 1953. *53.* 547—556.

COHN, W. E., Some results of the applications of ion-exchange chromatography to nucleic acid chemistry. *J. cell. comp. Physiol.* 1951. *38.* 21—40.

CRANE, R. K. and F. LIPMAN, The effect of arsenate on aerobic phosphorylation. *J. biol. Chem.* 1953. *201.* 235—243.

EADE, N. R., Differential sedimentation of noradrenaline in homogenates of adrenal medullary granules. *J. physiol.* (Lond.) 1956. *132.* 53 P.

EULER, U. S. v. and U. HAMBERG, Colorimetric determination of noradrenaline and adrenaline. *Acta physiol. scand.* 1949. *19.* 74—84.

FALCK, B., N.-Å. HILLARP and B. HÖGBERG, Content and intracellular distribution of adenosinetriphosphate in cow adrenal medulla. *Acta physiol. scand.* 1956. *36.* 360—376.

HERSHEY, A. D., J. DIXON and M. CHASE, Nucleic acid economy in bacteria infected with bacteriophage T 2. *J. Gen. Physiol.* 1953. *36.* 777—789.

HILLARP, N.-Å., Enzymic systems involving adenosinephosphates in the adrenaline and noradrenaline containing granules of the adrenal medulla *Acta physiol. scand.* 1958 a. *42.* 144—165.

HILLARP, N.-Å., Adenosinephosphates and inorganic phosphate in the adrenaline and noradrenaline containing granules of the adrenal medulla. *Acta physiol. scand.* 1958 b. *42.* 321—332.

HILLARP, N.-Å., Isolation and some biochemical properties of the catechol amine granules in the cow adrenal medulla. *Acta physiol. scand.* 1958 c. *43.* 82—96.

HILLARP, N.-Å., The release of catechol amines from the amine containing granules of the adrenal medulla. *Acta physiol. scand.* 1958 d. *43.* 292—302.

HILLARP, N.-Å, B. HÖGBERG and B. NILSON, Adenosinetriphosphate in the adrenal medulla of the cow. *Nature* (Lond.) 1955. *176.* 1032—1033.

HILLARP, N.-Å., S. LAGERSTEDT and B. NILSON, The isolation of a granular fraction from the suprarenal medulla, containing the sympathomimetic catechol amines. *Acta physiol. scand.* 1953. *29.* 251—263.

HILLARP, N.-Å. and B. NILSON, The structure of the adrenaline and noradrenaline containing granules in the adrenal medullary cells with reference to the storage and release of the sympathomimetic amines. *Acta physiol. scand.* 1954. *31.* Suppl. 113. 79—107.

LePAGE, G. A., in W. W. UMBREIT, R. H. BURRIS and J. F. STAUFFER, Manometric techniques and tissue metabolism. *Minneapolis. 2nd edition.* 1951. 185.

SCHÜMANN, H. J., The distribution of adrenaline and noradrenaline in chromaffin granules from the chicken. *J. Physiol.* (Lond.) 1957. *137.* 318—326.

SCHÜMANN, H. J., Die Wirkung von Insulin und Reserpin auf den Adrenalin- und ATP-Gehalt der chromaffinen Granula des Nebennierenmarks. *Arch. exp. Path. Pharmak.* 1958. *233.* 237—249.

WYATT, G. R., The purine and pyridine composition of deoxypentose nucleic acids. *Biochem. J.* 1951. *48.* 584—590.

Acta physiol. scand. 1959. *45*. 339—349.

From the Institute of Zoophysiology, University of Lund, Sweden.

Changes in Free Amino Acids and Proteins during Cell Growth and Synchronous Division in Mass Cultures of Tetrahymena Pyriformis.

By

ERIK CHRISTENSSON.

Received 14 November 1958.

Abstract.

CHRISTENSSON, E. G. Changes in free amino acids and proteins during cell growth and synchronous division in mass cultures of Tetrahymena pyriformis. *Acta physiol. scand.* 1959. *45*. 339—349. — The cellular divisions in mass cultures of the ciliate Tetrahymena pyriformis were synchronized through heat shocks. Changes in the protein metabolism during the heat treatment and the subsequent first synchronous division have been analysed by aid of one-dimensional paper chromatography of the free amino acids and nitrogen estimation of the soluble and insoluble proteins. Both the free amino acids and the total protein content increased about 3 times during shock treatment. Most individual free amino acids varied in amount in the same manner. At the higher temperature the protein synthesis showed to be blocked and as a result the synthetic activity proceeded in distinct waves corresponding to the individual shocks. The synthesis of soluble proteins showed a lag in time compared to the synthesis of the insoluble proteins. The protein synthesis was found to stop before division.

The discovery of a method for synchronizing division in Tetrahymena by heat treatment (SCHERBAUM and ZEUTHEN 1954, 1955) has provided an interesting material for physiological and biochemical studies of cell growth and cell division. Thus far this material has been utilized for analysis of cell growth through measurements of the cell size at different stages of heat treatment (SCHERBAUM 1956), for studies of the kinetic mechanism of synchronization (SCHERBAUM 1957), for estimation of the nucleic acid content and the ultraviolett susceptibility (IVERSON and GIESE 1957, SCHERBAUM 1957) and for studies of synchronous division in inorganic media (HAMBURGER and ZEUTHEN 1957). However cellular growth and division may be expected to involve changes in the protein metabolism. Investigation of such changes have not been made thus far. Therefore the present author has undertaken an analysis of the changes in free amino acids and proteins during these periods of the cell cycle.

Material and Methods.

Three-day-old stock cultures of Tetrahymena pyriformis grown under sterile conditions in proteose peptone medium with about 2×10^5 cells/ml were used for inoculation in a 1,500 ml culture flask. In each case was added two ml to 450 ml of medium. The divisions of the cultures were synchronized by exposing them during 8 hrs to temperature shifts of 30 min each alternating from $34 \pm 0.2°$ C to $29 \pm 0.2°$ C. This method of heat treatment was described by SCHERBAUM and ZEUTHEN (1955). Samples withdrawn from the medium with a pipette were centrifuged down rapidly, the medium sucked off and the cells washed several times in isotonic sodium-solution during 5—10 min until free from the medium. A definite fraction was fixed in 0.5 % formic acid for counting. The rest was rapidly centrifuged down and the material was frozen by dipping the tube in solid carbon dioxide. The proteins were separated into two fractions on the basis of solubility differences. To fractionate the proteins the extraction was carried out according to a method by KAVANAU (1954) with some modifications. This method includes separation of soluble from insoluble proteins in dilute salt-solution (0.1 M KCl — 0.011 M HAc), freeze-drying of the supernatant and extraction of the amino acids with ice-cold acetone (5 per cent 0.5 N HCl in acetone) from the freeze-dried material. All amino acids, some oligopeptides, glykosamine, among others, were retained quantitative (BOULANGER and BISERTE 1951) on cation resin, while polypeptides and sugars passed. It is important that free sugars appearing in large quantities are removed, as they make a good chromatographic separation impossible. The quantitative eluation of the

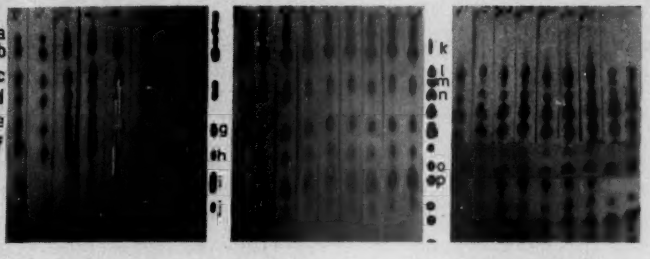

Fig. 1. The one-dimensional separation. To receive the ninhydrin-positive value on the same part of the standard curve the number of cells decrease from the left to the right.

A: Solvent phenol, both paper and solvent buffered at pH 12,

a) asparic acid, d) glycine,
b) glutamic acid, e) threonine,
c) serine, f) alanine.

B: Solvent m-Cresol, both paper and solvent buffered at pH 8.4,

g) valine, i) leucine-isoleucine,
h) methionine, j) phenylalanine.

C: Solvent, butanol-HAc-H_2O, 4:1:5,

k) cysteine, n) arginine,
l) lysine, o) proline (isatin coloured),
m) histidine, p) tyrosine.

amino acids was done by displacement with 1 N ammonia. After increasing the concentration of the amino acids the evaporation was continued to dryness in a vacuum exsiccator over sulphuric acid. Dissolution was carried out in 0.1—0.5 ml distilled water and the preparation was stored at — 20° C. Both one- and two-dimensional chromatograms were run at room temperature on filter paper (Whatman no 1). One-dimensional chromatograms were used for the quantitative estimation. Among different systems those of Mc Farren (1951) proved to be the best. Two of his systems have been used (Fig. 1 A, B), which made excellent separation of 10 different amino acids. Instead of Mc Farren's other systems, a solvent has been chosen which gave 6 further amino acids (Fig. 1 C). Drying of the chromatograms were carried out a 60° C. Each strip of sample was cut out and dipped into a butanol solution containing 0.4 per cent ninhydrin saturated with water. The dipping technique gave greater optical density (Mc Farren 1952) than the spraying technique. The development of ninhydrin colour and dissolving the colour with 71 per cent ethanol from the cut spot was carried out in accordance with an earlier described method (Kay, Harris and Entenman 1956). All amino acids were stained with ninhydrin, with the exception of proline, which was stained with isatin. The density was measured at 575 mμ in a Klett Summerson photometer. The

Table I.

Some values of the free acid content (10^{-11} g/cell) are represented from some different shocks. Each figure is the mean value of five measurements.

	Before heat treatment	During heat treatment						After heat treatment	
		Before 4:th shift	After 4:th shift	Before 6:th shift	After 6:th shift	Before 8:th shift	After 8:th shift	Before division	After division
Alanine	2.67	2.62	3.82	4.00	5.80	6.23	6.90	7.20	4.01
Arginine	1.91	2.78	3.40	3.99	4.55	4.26	4.54	5.13	3.92
Aspartic acid .	1.21	1.00	1.33	1.33	2.53	2.20	2.46	4.20	2.10
Cysteine	0.26	0.24	0.60	0.36	0.73	0.60	0.72	0.73	0.60
Glutamic acid	2.98	3.48	6.78	6.35	8.08	9.16	9.71	11.9	6.43
Glycine	1.89	1.28	1.92	2.07	2.62	3.83	4.31	5.64	2.66
Histidine	0.45	0.70	1.16	0.95	1.16	1.38	1.38	2.86	1.63
Leucine + Isoleucine ..	4.27	4.46	6.03	8.85	11.0	10.5	10.2	12.5	7.22
Lysine	2.43	7.31	10.0	8.85	11.0	10.3	10.3	13.1	6.64
Methionine ...	0.27	0.18	0.22	0.22	0.37	0.75	0.75	0.37	0.36
Phenylalanine	1.29	1.24	1.50	1.74	2.23	3.72	3.06	4.72	2.81
Proline	1.18	1.15	1.04	0.87	1.27	1.09	1.27	1.15	1.09
Serine	1.20	1.05	1.22	1.01	1.32	1.84	2.16	3.84	1.84
Threonine	1.29	1.37	1.67	1.49	1.73	2.98	3.10	3.70	2.86
Tyrosine	1.15	0.99	1.81	2.08	2.54	2.90	2.72	3.62	2.54
Valine	1.19	1.34	1.44	1.70	2.23	3.40	3.40	3.41	2.58
	25.64	31.19	43.94	45.86	59.16	65.14	66.98	84.07	49.29

quantity of each amino acid could be calculated from a carefully made standard curve with an error of 4 per cent. The nitrogen content of insoluble and soluble proteins has been estimated according to a method described by BOISSONNAS and HASELBACH (1953).

Results.

The two-dimensional chromatograms.

In accordance with earlier observations of untreated cells of Tetrahymena (WU and HOGG 1956) all 16 amino acids were found. On two-dimensional chromatograms the spots of all 16 could be identified. Two spots near lysine and orginine were assumed to be possible ornithine and citrulline. A yellow spot, that disappeared on hydrolysis was probably glycosamine.

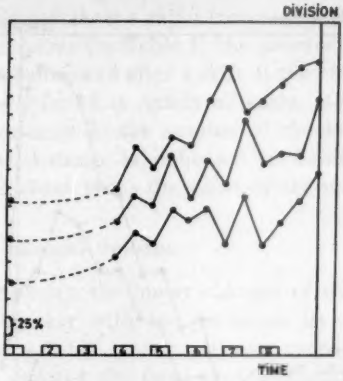

Fig. 2. The variations in optical density of ninhydrin in per cent per cell in three different series from the 4th shock up to division. The squares on the horisontal axis represent the different shocks. The length of each shock = 30 min.

The total ninhydrin-positive variation.

The curves for the variation of the total ninhydrin-positive value of the amino acid fraction during the last four temperature shocks, up to the first synchronous division in three different series, are shown in Fig. 2. The values represent the percentage of the value in an untreated cell. The curves are seen to change rhythmically until the beginning or the end of the seventh shift. Then after a short declination they raise again up to the time of synchronous division. This tendency to drop at the end of heat treatment is significant but can be somewhat displaced in time.

The changes of the free amino acids.

Changes in free amino acid composition during cell growth and synchronous division are listed in Table I. Each figure is the mean value in general of five measurements. In Fig. 2, the total ninhydrin-positive value, is immediately before division, 2—3 times greater than that of the untreated cell, while the corresponding increase for the free amino acids is about 3—4 times, Table I. This indicates the amount of oligopeptides or glycosamine is constant or decreased. Also the ninhydrin-positive value of the

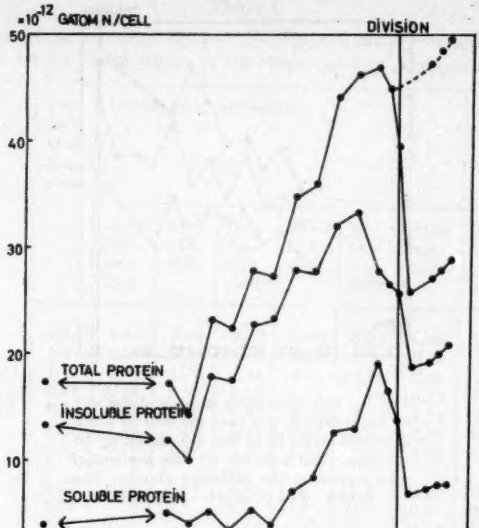

Fig. 3. The variations in total, insoluble and soluble protein content and the free amino acid amount during heat treatment and first synchronous division. Each point in the curves is in general the mean value of 5 measurements. The thermal shocks employed are represented on the horizontal axis as a series of small squares. The time length of each shock = 30 min. The time interval between the shocks is = 30 min. •–•–•–•–•. This represents the hypothetical growth that would have resulted if no division had taken place. This curve was calculated from the division index and the amount of protein in the divided cell.

polypeptides that passed the cation resin showed a decrease during heat treatment when compared to the value of the untreated cell. From Table I it may be that lysine, histidine and glutamic acid increased their content during heat treatment more than the rest. On the other hand proline and methionine remained constant.

Aspartic acid did not show a rapid increase until after heat treatment. If one compares (in Table I) the amount of the individual free amino acids before and after a shift at the higher temperature, a great increase is found in nearly all cases. However this effect drops off continuously as the number of shocks increases. After the synchronous division as indicated in Table I the amounts fall but are still about twice the value of the untreated cell.

The soluble and insoluble proteins.

In Fig. 3 are shown the molar changes of the sum of the 16 amino acids, together with the variation in nitrogen content of insoluble and soluble proteins during growth. On the basis of the nitrogen content the total protein amount per cell is increased about 300 per cent during heat treatment. This means that during the period of treatment there was high synthetic activity. It is of great interest to note that the synthetic activity is not constant but stops completely during the first shocks and partially during the last, so that growth goes on in distinct waves. The protein synthesis stops before the synchronized division. After division, this synthesis proceeds but at a lower speed than during heat treatment up to the second division. If soluble and insoluble proteins are compared the same cyclic variations occur in both, but distinct differences are observed. These differences are mainly due to the fact that the synthetis of the soluble proteins shows a lag in comparison to insoluble proteins.

Discussion.

SCHERBAUM and ZEUTHEN (1954) have shown that when the temperature is shifted from the optimum of 29° C to the sublethal value 34° C, growth and division are affected so as to bring the cells into synchrony. They suggest that sublethal temperature prevents growth. This assumption is based on an earlier observation (PHELPS 1946), that sublethal temperatures (32—34° C) partially or completely block growth of Tetrahymena cultures. At the sublethal temperature the organism does not divide, possibly because som mechanism necessary for division is blocked. On return to 29° C the cells eventually get ready to divide again, but before they can do so, they receive another shock by which

the preparatory work for division is undone. At the end of heat treatment they found that the mean size of the cells had increased about 2—3 times.

I have tried to analyse growth in terms of protein synthesis from the fourth shock, when the division index approaches zero, up to the synchronous division and the time immediately after it. As seen from Fig. 3 protein synthesis starts very quickly after return to the optimum temperature. Under sublethal periods there is a complete inhibition up to about the seventh shock and then a partial block. The above shows that the recovery and block mechanisms for growth act immediately. To what extent the amino acid adsorption is dependent on the temperature shifts is difficult to answer. But following general physical laws, the adsorption should depend both on temperature and on free sites for the disposition of the amino acids. In living material the free amino acid content should depend not only on the adsorption from the medium but also the synthetic activity and the degradation of proteins. By blocking the protein synthesis in Esherischia coli (MANDELSTAM 1958) it was found, that a definite number of sites were available for the free amino acids. This number was found to be correlated with the content of protein and nucleic acids. The relatively small temperature fluctuation probably plays a secondary role in adsorption.

The observation that both lysine and glutamic acid increase at the same rate might be expected since the balance between basic and acid free amino acids would be maintained. It is somewhat surprising to find that aspartic acid seems to raise rapidly immediately after heat treatment. This can depend on nitrogen transfer, since nitrogen is to be incorporated readily from aspartic acid into nucleotides (LAGERKVIST, REICHARD and EHRENSVÄRD 1951). Proline and methionine remained nearly constant. Alanine and arginine showed less increase than the average for all the amino acids. This may perhaps indicate the incorporation of these in proteins of protamine or histone types rich in arginine, alanine and proline.

The protein metabolism during heat treatment up to division can be divided into three periods. The first reaches up to about the seventh shock. This period of time seems to be characterized by rapid synthesis of insoluble structural proteins during optimal temperature periods. The amount of soluble proteins is kept at a low level probably because of a rapid incorporation into the in-

soluble state. The block of the synthetic acitivity under sublethal temperature periods is complete and some degradation is indicated in both soluble and insoluble proteins. The amount of free amino acids rise stepwise. During optimal periods there is a decrease in the amount of free amino acids owing to the great synthethic activity.

The second period of protein metabolism should include the last heat shocks during heat treatment. The higher temperature has here lost much of its blocking effect on the protein synthesis and a protein syntheis occurs also during the heat shocks. This may be due to an increased protein stabilisation, or the phenomenon of adaptation common in biological material. Towards the end of this period synthesis of insoluble proteins seems to be finished. In addition there is a rapid increase in the amount of soluble proteins. The high synthetic activity independent of the temperature changes makes a significant decrease in the free amino acid content during this period. In some cases there was noted a raise during the period of optimal temperature between the 8th and 7th shock both in free amino acid and the protein content (Fig. 2, 3). Whether this is caused by increased degradation of peptides into amino acids or an experimental error is an open question.

The third part of the protein metabolism includes the period from the end of heat treatment up to the first synchronous division, when the protein synthesis has stopped and when even a fall in the protein content can be seen. The observation that protein synthesis stops before division agrees well with earlier investigations. PRESCOTT found (1955) that in different amoebae growth stops about 4 hours before mitosis and ZEUTHEN has observed (1953) in a single Tetrahymena in a microspirometer, that the rate of respiration is rhythmic, which should correspond to a synthetic and a divisional phase. From Fig. 3 it may be observed that before division insoluble proteins break down into soluble. This may indicate, that before division the structural proteins of the cell are at least to some degree broken down.

Summary.

1. The variation in amount of 16 free amino acids and of soluble and insoluble proteins were studied during heat treatment and the first synchronous division in Tetrahymena pyriformis.

2. The free amino acid content was found to raise rhythmically during heat treatment but showed a significant decrease during the last shocks.

3. The free amino acid content immediately before division was about 3 times that of the untreated cell.

4. Individual free amino acids varied in amount in the same manner with some few exceptions.

5. The total protein amount increased about 3 times during heat treatment. The synthethic activity during heat treatment went on in distinct waves corresponding to the individual shocks.

6. During the sublethal temperature periods synthesis was completely blocked up to the last shocks when this blocking occurred to a lesser extent.

7. The synthesis of soluble proteins showed a lag in time compared to the the synthesis of the insoluble proteins.

8. The protein synthesis was found to stop before division.

9. After the synchronous division the synthesis started again and proceeded at a lower speed.

I wish to express my gratitude to Dr. Ivar Agrell, Director of the Institute of Zoophysiology, who proposed this investigation, for his instructive discussions and helpful criticism. I also wish to express my gratitude to Dr. Nils Olof Lindh for his valuable advice.

References.

Boissonnas, R. A. and Haselbach, C. H., Microdosage colorimétrique en série de de l'azote protéique. *Helv. chim. Acta* 1953. *73.* 576—581.

Boulanger, P. and Biserte, G., Chromatographie sur papier des acides amine's et polypeptides des liquides biologiques. *Bull. Soc. Chim. biol.* (Paris) 1951. *33.* 1930—1939.

Hamburger, K. and Zeuthen, E., Synchronous division in Tetrahymena pyriformis as studied in an inorganic medium. *Exp. Cell. Res.* 1957. *13.* 443—453.

Iverson, R. M. and Giese, A. C., Nucleic acid content and ultra violet susceptibility of Tetrahymena pyriformis. *Exp. Cell. Res.* 1957. *13.* 213—223.

Kavanau, J. L., Amino acid metabolism in the early development of sea urchin. Paracentrotus lividus. *Exp. Cell. Res.* 1954. *7.* 530—557.

Kay, R. E., Harris, D. C. and Enteman, C., Quantification of the ninhydrin color reaction as applied to paper chromatography. *Arch. Biochem. Biophys.* 1956. *63.* 14—25.

Lagerkvist, V., Reichard, P. and Ehrensvärd, G., Aspartic acid as a precursor for ribonucleic acid pyrmidines. *Acta chem. scand.* 1951. *5.* 1212.

Mandelstam, J., The free amino acids in growing and non-growing populations of Escherichia coli. *Biochem. J.* 1958. *69.* 103—110.

Mc Farren, F., Buffered filter paper chromatography of the amino acids. *An. Chem.* 1951. *23.* 168—174.

Mc Farren, F. and Mills, J. A., Quantitative determination of amino acids on filter paper chromatograms by direct photometry. *An. Chem.* 1952. *24.*6 50—660.

PHELPS, A., Growth of protozoa in pure culture. *J. exp. Zool.* 1946. *102*. 277—292.

PRESCOTT, D. M., Relations between cellgrowth and cell division. *Exp. Cell. Res.* 1955. *9*. 328—337.

SCHERBAUM, O. and ZEUTHEN, E., Induction of synchronous cell division in mass cultures of Tetrahymena pyriformis. *Exp. Cell. Res.* 1954. *6*. 221—227.

SCHERBAUM, O. and ZEUTHEN, E., Temperature-induced synchronous division in the ciliate protozoa Tetrahymena pyriformis growing in synthetic and proteose — peptone media. *Exp. Cell. Res.* Suppl. 1955. *3*. 312—325.

SCHERBAUM, O., Cell growth in normal and synchronously dividing mass cultures of Tetrahymena pyriformis. *Exp. Cell. Res.* 1956. *11*. 464—476.

SCHERBAUM, O., Studies of the mechanism of synchronous cell division in Tetrahymena pyriformis. *Exp. Cell. Res.* 1957, *13*. 11—23.

SCHERBAUM, O., The content and composition of nucleic acids in normal and synchronously dividing mass cultures of Tetrahymena pyriformis. *Exp. Cell. Res.* 1957. *13*. 24—30.

WU, C. and HOGG, J., Free and nonprotein amino acids in Tetrahymena pyriformis. *Arch. Biochem. Biophys.* 1956. *64*. 70—77.

ZEUTHEN, E., Growth as related to the cell cycle in single-cell cultures of Tetrahymena pyriformis. *J. Embryol. exp. Morph.* 1953. Part 3. 239—249.

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From the Central Laboratory and the Department of Surgery II, University of Gothenburg, Sahlgrenska sjukhuset, Gothenburg, Sweden.

The Behaviour of Potassium in Rat Liver Cells at Physiological Variations of the Glycogen Content.

By

Y. EDLUND, B. ISAKSSON and H. SUNZEL.

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Abstract.

EDLUND, Y., B. ISAKSSON and H. SUNZEL. The behaviour of potassium in rat liver cells at physiological variations of the glycogen content. *Acta physiol. scand.* 1959. *45.* 350—362. — The amounts of glucose, water and potassium entering rat liver cells in vivo were calculated in 34 animals. The estimations of these components and of fat, nitrogen and chloride were made on the homogenized liver, and the results related to the glycogen content. The calculations were based on the assumption that the absolute amount of nitrogen per cell remained unchanged during the experimental conditions. If 115—175 meq potassium are required per litre water for maintaining osmosis, "extra" potassium ranging from 50 to 25 per cent of the total was found to enter the cell. The possible explanations for the entrance of the "extra" potassium are briefly discussed.

A connection between potassium and carbohydrate metabolism is generally accepted. Numerous papers have dealt with this problem but the nature of the connection is still lively discussed.

Most opinions about the relationship are based upon experiments either on isolated cells, tissue slices or microorganisms. All these studies have shown that potassium enters the cell during glycogen synthesis. The mechanism has been explained in different ways: (1) potassium takes an active part in the formation of glycogen (PULVER and VERZÁR 1940 — yeast cells), (2) potassium is passively exchanged with hydrogen and/or sodium ions (CONWAY, BRADY and CARTON, 1950 — yeast cells), (3) potassium is transferred "into complexes with special cellular proteins" (VERZÁR 1952, p. 442), (4) potassium is incorporated into the phosphorylation of the adenylic system (BOYER, LARDY and PHILLIPS 1943 — isolated muscle).

There are, however, only few investigations on the living organism. FENN (1939) found in rat liver glycogen to be deposited with the same amount of water, potassium and acid-soluble phosphorus as was associated with the other solids. He also pointed out that only a very small fraction, if any, of the potassium could be in the form of K-hexosephosphate. Recently, AHRENSMEYER and MOLINSKI (1954) have supposed that the uptake of potassium plays a role only in the regulation of the intracellular osmosis.

The clinical approach to the problem is mainly based on observations of the serum potassium. The administration of glucose and insulin has been reported to give a rapid but transient decrease in serum potassium (GROEN *et al.* 1951).

In the present paper we report a study on the content of potassium and water in rat liver cells at different glycogen levels and calculations concerning the distribution of the potassium entering the cell during glycogen synthesis.

Material and methods.

Thirty-four male albino rats weighing 240—360 g and of the same strain were used. They were maintained on a diet of ordinary mouse bread and water *ad lib.*

In order to get different glycogen levels, 26 animals were killed in two different phases of the diurnal rhythm — 16 in the assimilation phase (8 *a.m.*) and 10 in the dissimilation phase (8 *p.m.*). The remaining 8 rats were killed at 8 *a.m.* after 24 hours of fasting.

The liver was removed in toto as soon as the animal had been killed by decapitation. Two small liver pieces (200—500 mg) were

blotted free of blood with filter paper, the first piece was weighed in 5 ml 5 per cent trichloroacetic acid (TCA), the second in 5 ml distilled water. The following homogenization in Potter-Elvehjem homogenizers was regularly completed within 5 min after the death of the animal. The remaining liver pieces and the carcass were then weighed.

Glycogen was determined according to VAN DER VIES (1954) slightly modified: The TCA homogenizate was centrifuged, the residue washed once with 5 ml TCA. The combined centrifugates were diluted to 20.0 ml and 2.00 ml transferred to a ground glass stoppered tube, containing 2.00 ml 10 N KOH. The tube was immersed in boiling water for 1 hr, cooled and 1.00 ml glacial acetic acid added. The mixture was diluted to 15.00 ml with water and 2.00 ml transferred to a carefully cleaned test-tube, of standard size. A glass-tube, connected with an air-pressure system was immersed into the test-tube and a stream of water-rinsed air was led through the solution with a constant velocity. After about 1 min 4.00 ml of a 0.1 per cent solution of anthrone in concentrated sulphuric acid was added by means of a quick-emptied syringe. The air-bubbling continued for 35 sec, the tube was then left for spontaneous cooling down to room temperature. The absorbance was measured within one hour in a Beckman B spectrophotometer in 1 cm cuvettes at 625 mμ against water.

Total water was determined by freeze-drying of the water homogenisate in an Edward Centrifugal Freeze Dryer, L.C.5.

Part of the lyophilized residue was subjected to acid digestion and *nitrogen* determined with the Kjeldahl technique.

Another part of the lyophilized residue was extracted 2 hours with boiling ethanol-ethyl ether (3 : 1) for determination of *total fat*. The extract was filtered, evaporated in vacuo, the residue dissolved in chloroform and filtered into a weighing glass, the chloroform evaporated on water bath and the fat content determined by weighing. The error in the gravimetric determination of fat is thus markedly reduced as the extraction was performed on a water-free material.

Potassium was determined by flame photometry on the TCA-extract, as it was found to be quantitatively extracted by the acid, no wet combustion of the organic material or nitric acid extraction being necessary (no measurable K was found in the residue from the TCA-extraction).

Chloride was determined by titration with mercury nitrate in

the modifications described by BRUN (1949) on a water extract of the rest of the lyophilized material. The results obtained seem, however, not quite satisfactory — the amount of material available being rather small.

Blood samples were collected from the neck when bleeding the animal. In several samples, however, a rather pronounced hemolysis occurred. The material was therefore supplemented by samples taken by heart puncture on animals, maintained in the same way. No significant differences appeared between the blood values found on test animals and on the heart punctured animals. Potassium was determined by flame photometry, chloride by titration with mercury nitrate, specific gravity by weighing and total water by freeze drying.

All reagents used were of analytical grade, blanks and standards were run in every serial determination.

Calculations.

The analytical values obtained were subjected to the following calculations:[1]

A. *Per kg fat-free wet liver:* Glycogen /G/, nitrogen /N/, total water $/H_2O/_t$ and intracellular water $(H_2O)_i$ were calculated in g, total potassium $/K/_t$, intracellular potassium $(K)_i$ and total chloride $/Cl/_t$ in meq per kg fat-free wet liver.

For the calculations of $(H_2O)_i$ and $(K)_i$ the formulas given by HASTINGS and EICHELBERGER (1937) were applied, using the chloride space as a measure of extracellular water.

The weight of the fat-free liver /L/ was calculated in per cent of total body weight.

B. *Per single liver cell:*[2] The following calculations were undertaken in order to get an approximate impression of the cell content, facilitating the interpretations of the results:

1. Cell weight (W)

$$(W) = \frac{/N/_I}{/N/} \times [W]_I \quad \text{B.1.}$$

[1] The brackets / / are used around figures obtained by analysis or simple calculations; ( ) around figures obtained from calculations based on assumptions and [ ] design constants.

[2] The calculations are based on the assumption that the absolute amount of nitrogen per single cell remains unchanged during glycogen accumulation, see page 360.

where the constant $[W]_I$ = the found average cell weight in the glycogen-"free" group I,[1]
$/N/_I$ and $/N/$ = the found average value for nitrogen in group I and the group under consideration (group II—IV) respectively.

2. Number of cells (C) per kg fat-free wet liver

$$(C) = \frac{/N/}{/N/_I} \times [C]_I \quad \text{B.2.}$$

where the constant $[C]_I$ = the average number of cells in group I.

3. The amount of glycogen $(G)_c$, intracellular water $(H_2O)_c$ and potassium $(K)_c$ per cell was obtained by dividing $/G/$, $(H_2O)_i$ and $(K)_i$ by (C). B.3.

4. The increase per cell of glycogen $\Delta\ (G)_c$, intracellular water $\Delta\ (H_2O)_c$ and potassium $\Delta\ (K)_c$ was obtained by subtracting the calculated figures from B. 3. by the corresponding values for group I. B.4.

5. The part played by potassium in maintaining osmolarity during water accumulation $\Delta\ (K)_{osm}$

$$\Delta\ (K)_{osm} = \frac{\Delta\ (H_2O)_c \times [K]_{osm}}{1{,}000} \quad \text{B.5.}$$

where $[K]_{osm}$ has arbitrarily been chosen as 175, 145 and 115 mOsm/kg intracellular water.

6. The "extra" potassium entering the cell $\Delta\ (K)_{ex}$

$$\Delta\ (K)_{ex} = \Delta\ (K)_c - \Delta\ (K)_{osm} \quad \text{B.6.}$$

Results.

In the fasted animals liver *glycogen* ranged 1—7 g per kg wet tissue (M : 2.5 g), the corresponding figures for the animals killed at 8 *p.m.* being 5—7.6 g (M : 32.7 g) and for those killed at 8 *a.m.* 16—82 g (M : 40.0 g). No significant difference was thus found between those killed at the different phases of the diurnal rhythm. By rearrangement of the animals according to increasing level of liver glycogen, four different groups with rather well separated glycogen ranges were obtained (Table I). The Roman figures

[1] The different groups are defined on page 355.

I—IV are used to refer to the respective groups, Group I having the lowest glycogen range.

The analytical values are collected in Table I. *Total fat* in g per kg dry tissue was probably higher in Group I ($P < 0.05$) than in the other groups, *nitrogen* in g per kg dry tissue decreased with increasing glycogen values, the differences between Groups II, III and IV being significative ($P < 0.01$). No differences between the groups were found for water, potassium and chloride.

In the following calculations it thus seemed more correct to use the over-all mean for water, potassium and chloride respectively instead of the separate group mean, and for total fat the over-all mean of Groups II, III and IV.

Serum potassium was slightly higher in Group I (M : 4.6 meq/l) than in the other groups (M : 4.0). No differences were found for the other components studied in serum, hence they are not recorded here.

Calculations per kg fat-free wet liver (Table II). The significant differences for *nitrogen* between Groups II, III and IV persisted due to the fact that the amount of fat was "constant" throughout the groups.

The average intracellular water content amounts to 64 per cent of total water, intracellular *potassium* was 102 meq/kg fat-free liver or 210 meq/kg intracellular water. The *quotient* K/N increased with increasing glycogen values, the lowest figure being 3.06.

The table also includes the weight of the fat-free liver in per cent of total body weight. The weight in Group II and III is probably higher than in Group I ($P < 0.05$), and in Group IV significantly higher than in Group II and III ($P < 0.01$).

Calculations at the cellulary level (Tables III and IV). When assuming the absolute amount of *nitrogen* per single cell being constant and taking weight and number of cells in Group I as unite of reference the following was found:

The calculated increase in *cell weight* (formula B.1) fitted rather well with the found increase in liver weight. The increase is insignificant in Group II but pronounced at higher glycogen levels (Group III and IV).

The increase in *potassium* per cell in Group III and IV was about 25 per cent higher (= "extra" potassium — Formula B.6) than the amount calculated to accompany the water entering the cell at a concentration of 175 meq/l, and about 50 per cent higher if the corresponding concentration was 115 meq/l.

Table I.

Analytical data from rat livers, grouped according to different glycogen levels.

Group	No of animals	Glycogen g	Total fat g		Nitrogen g		Total water g		Total potassium meq		Total chloride meq	
		per kg wet tissue	per kg dry tissue				per kg wet tissue					
		M±E(M) Range	M±E(M) Range	S.D.	M±E(M) Range	S.D.	M±E(M) Range	S.D.	M±E(M) Range	S.D.	M±E(M) Range	S.D.
I	10	3.4±0.9 1–8	250±13.0 208–323	38.8	101±2.0 90–110	5.8	695±7.1 661–716	18.8	92±2.2 83–107	6.9	30±2.1 20–36	5.9
II	11	23±1.3 16–27	208±5.8 177–245	19.2	98±1.9 87–105	6.0	712±5.8 683–742	18.2	96±3.1 84–116	10.4	30±1.5 23–36	4.6
III	7	43±3.5 30–52	214±10.4 181–243	25.4	89±2.1 78–94	5.6	681±10.0 659–726	24.4	99±6.3 79–115	15.4	32±1.7 27–37	4.1
IV	6	70±3.4 62–82	206±8.4 173–234	20.7	79±2.5 72–85	5.6	689±6.2 670–706	15.2	99±4.5 85–113	11.0	32±1.3 29–37	3.1
I—IV	34	—	210±4.9 173–245 (II–IV)	20.5	—	—	697±4.0 659–742	21.8	96±1.8 83–116	10.6	30.5±0.8 20–37	4.6

Table II.

Calculations: Fat-free liver.

Symbol	/L/		/G/	/N/	/H_2O/$_t$	(H_2O)$_i$	/K/$_t$	(K)$_i$		K/N
	Weight of fat-free liver in per cent of total body weight		Glycogen	Nitrogen	Water		Potassium			
					Total	Intra-cellular	Total	Intracellular		
			g	g	g	g	meq	meq	meq	meq
Group	M±E(M) Range	S.D.	per kg fat-free wet liver						per kg intracel-lular water	per g nitrogen
I	2.56±0.10 2.23–3.04	0.28	4	33.3	755	485	103	102	210	3.06
II	2.80±0.14 2.24–3.35	0.37	25	31.9	745	475	103	102	215	3.20
III	2.89±0.05 2.68–3.04	0.13	46	28.8	745	475	103	102	215	3.54
IV	3.32±0.14 2.90–3.59	0.30	75	25.6	745	475	103	102	215	4.00

Table III.

Calculations: The cellulary level.

Symbol	$(L)_B$	(W)	(C)	$(G)_c$	$(H_2O)_c$	$(K)_c$
	Liver weight	Cell weight	No. of cells per kg liver	Glycogen g	Intracellular water g	Intracellular potassium meq
Group	$x \frac{1}{[L]_I}$	$x[C]_I$	$x \frac{1}{[C]_I}$	per cell x $[C]_I$		
I	1.00	1.00	1.00	4	485	102
II	1.09	1.05	0.95	26	500	107
III	1.13	1.16	0.86	54	550	118
IV	1.30	1.30	0.77	98	615	133
Formula	—	B. 1	B. 2	B. 3	B. 3	B. 3

Discussion.

The purpose of killing the animals at different phases of the diurnal rhythm was to achieve different liver glycogen levels. As that for unknown reasons failed, the animals were rearranged as to the actual concentrations found.

The analytical results of the present investigation confirm earlier reports (FENN 1939, AHRENSMEYER and MOLINSKI 1954) on the simultaneous entrance of glycogen, water and potassium into the liver.

The increase of liver weight and relative decrease in nitrogen concentration exceeds markedly what could be expected from merely an increase in glycogen. The constant relationship between water and solids at different glycogen levels is additional evidence for the water entrance. As to the water entering the liver the partition between the extra- and intracellular compartment was the same as for the "stationary" water, which is in accordance with FENN (1939). Despite the fact that only small amounts of material were available for the chloride analyses, our figures for the partition also agree with those reported by him. In recent reports the extracellular compartment has been found to be slightly lower than ours, and about 5—15 per cent of the chloride has been reported to be intracellular. The error in our determination may, however, not be great, and may by no means impair the other calculations performed.

Table IV.

Calculations of the increase per cell of glycogen, water and potassium, and of the partition of the potassium increase between osmotic active and inactive ("extra") phases at different assumed levels for potassium concentration in the water entering the cell.

Symbol	$\Delta (G)_c$	$\Delta (H_2O)_c$	$\Delta (K)_c$	$\Delta (K)_{osm}$				$\Delta (K)_{ex}$		
	Increase of			meq potassium engaged by $\Delta (H_2O)_c$ if contributing to				meq "extra" potassium at		
	Glycogen g	Intracellular water g	Intracellular potassium meq	175 mOsm/kg	145 mOsm/kg	115 mOsm/kg		175 mOsm/kg	145 mOsm/kg	115 mOsm/kg
Group	per cell x $[C]_I$			per cell x $[C]_I$				per cell x $[C]_I$		
II	22	15	5	2.5	2.0	1.5		2.5	3.0	3.5
III	50	65	16	11.5	9.5	7.5		4.5	6.5	8.5
IV	94	130	31	23.0	19.0	15.0		8.0	12.0	16.0
Formula	B. 4	B. 4	B. 4	B. 5				B. 6		

As to potassium, the observed constancy of its concentration means a cellular uptake, running parallel with that of glycogen and water.

The calculations at the cellulary level were performed in order to obtain informations about the amount of potassium, water and glucose (as glycogen) entering the cell. All these calculations were based on the assumption that the *absolute* amount of nitrogen constituting the single cell remains unchanged (or diminished insignificantly) during the experimental conditions applied. The decrease of the *relative* amount of nitrogen when glycogen and water increase has therefore been taken as a measure of the increase in cell weight. The validity of this assumption is supported by the observed increase in liver weight, which nearly coincides with the increase (in cell weight) calculated from the nitrogen figures. Furthermore, there appeared no significant difference in the nitrogen content of the liver between group I (fasted animals) and group II (fed animals — low glycogen). Finally, if we apply the figure for the amount of water deposited along with glycogen given by FENN (2.33 ml water per 1 g glycogen) the increase in cell weight will be 10, 20 and 34 per cent for Group II, III and IV respectively, which are well fitted in with our calculated values.

As to the observed total increase of intracellular potassium (being of the same percentage degree as the increase of cell weight) one major function is certainly to contribute in maintaining the internal osmotic pressure, when cell water increases. An alternative appreciable decrease of the osmosis or an inclusion of the water into organic molecular structures without solutes seems us doubtful. We have calculated the amount of potassium osmotically engaged by the increase of the intracellulary water. The calculations are, however, impaired for different reasons: (1) other cations, *e. g.* sodium and magnesium, may be involved simultaneously in unknown quantities, (2) a shift in the proportion between osmotically active and inactive solutes inside the cell may occur (DANOWSKI 1951), (3) the osmotic pressure may change during the metabolic processes.

No exact figures are known for the part of the intracellulary potassium being osmotically active. We have therefore calculated with different figures, 175, 145 and 115 meq/l respectively. We regard 175 meq/l to be in the maximal range, representing the total amount of cations contributing to the internal osmotic

pressure according to GAMBLE (1954, p. 9). He claims that 145 meq/l is due to potassium, WEISBERG (1950, p. 34) reports the figure 115 meq/l, hence we have also chosen these figures, representing a medium and a minimum value.

In every calculation there appeared an "*extra*" *potassium*, amounting to 25—50 per cent of the total increase, which seemed to have entered the cell regardless of the water entrance. This "extra" potassium will reach or exceed the 50 per cent level if (1) appreciable amounts of other cations are involved for maintaining osmosis, if (2) osmotically inactive ions are liberated within the cell, or if (3) the intracellular osmosis will decrease. On the other hand the "extra" potassium will reach zero if a greater part or all potassium is osmotically active, it means either an increase of the intracellular osmosis or a maintaining of an initially high degree of hyperosmolarity. According to modern theories, based on *in vitro* experiments, there is a certain hyperosmosis inside the cell (OPIE 1954). It seems, however, doubtful that the hyperosmolarity in the liver cells should be so pronounced that it allows the entire amount of cell potassium to be osmotically active, thus alone responsible for 210—215 mOsm/kg cell water.

AHRENSMEYER and MOLINSKI (1954) also observed a constancy of the relations potassium/dry weight and potassium/wet weight respectively, at different glycogen levels. They postulated that potassium entered the cell in amounts only sufficient to maintain the internal osmosis ("Ionenmilieu"). Their argumentation was, however, based on the wrong statement that the amount of protein (and thus the amount of potassium associated with protein) remained constant. This may be true for the particular cell only, but not for the tissue unite under their examination, as its amount of protein decreases (due to cell swelling). Their figures, as well as those of FENN (1939), may therefore be as indicative as ours for the appearance of "extra" potassium.

What is the mechanism for the entrance of the "extra" potassium? There has never been shown evidences for a chemical association between potassium and glycogen, nor between potassium and ATP or other polyphosphates, all known to have relatively high dissociation constants. No "extra" potassium is thus needed for special functions in the carbohydrate metabolism. On the other hand, an active secretion of sodium or hydrogen ions in exchange with potassium may explain the appearance of the "extra" amount. The same is true for potassium forming a com-

plex with certain anionic sites on the cell surface (ROTHSTEIN 1955). As far as we can see, the advocators of these mechanisms, have not taken into sufficient account the appreciable amount of potassium being engaged by the water for isoosmosis. We propose that only the rest, the "extra" potassium may be available for any of these processes, but our experimental conditions do not allow any conclusions about the particular mechanism involved.

Thanks are due to Mrs. S. BENSON and Mrs. B. HALLENBERG for valuable technical assistance.

References.

ARENSMEYER, E. and H. MOLINSKI, Über den Gehalt an Glykogen und Kalium in Blut, Leber, Muskel und Niere von Ratten nach Hunger, Glucosemast, Insulin- und Adrenalineinwirkung. *Z. ges. exp. Med.* 1954. *122*. 564—577.

BOYER, P. D., H. A. LARDY and P. H. PHILLIPS, Further studies on the role of potassium and other ions in the phosphorylation of the adenylic system. *J. biol. Chem.* 1943. *159*. 529—541.

BRUN, C., A mercurimetric method for the determination of chloride. *Nord. Med.* 1949. *42*. 1774—1776.

CONWAY, E. J., T. G. BRADY and E. CARTON, Biological production of acid and alkali. *Biochem. J.* 1950. *47*. 369—374.

DANOWSKI, T. S., Newer concepts of the role of sodium in disease. *Am. J. Med.* 1951. *10*. 468—480.

FENN, W. O., The deposition of potassium and phosphate with glycogen in rat liver. *J. biol. Chem.* 1939. *128*. 297—307.

GAMBLE, J. L., Chemical Anatomy, Physiology and Pathology of Extracellular Fluid. *Cambridge, Mass., Harvard University Press.* 1954. p. 9.

GROEN, J., A. F. WILLEBRANDS, C. E. KAMMINGA, H. K. VON SCHOTHORST and E. G. GODFRIED, Effects of glucose administration on the potassium and inorganic phosphate content of the blood serum and the electrocardiogram in normal individuals and in non-diabetic patients. *Acta med. scand.* 1951. *141*. 352—366.

HASTINGS, A. B. and L. EICHELBERGER, The exchange of salt and water between muscle and blood. *J. biol. Chem.* 1937. *117*. 73—93.

OPIE, E. L., Osmotic activity of liver cells and melting point of liver. *J. exp. Med.* 1954. *99*. 29—41.

PULVER, R. and F. VERZÁR, Connection between carbohydrate and potassium metabolism in the yeast cell. *Nature* (Lond.). 1940. *145*. 823—824.

ROTHSTEIN, A., Relationship of the cell surface to electrolyte metabolism in yeast. Electrolytes in Biological systems by A. M. SHANES. *Baltimore, Waverly Press, Inc.*, 1955. p. 65.

VERZÁR, F., The connection of carbohydrate and potassium metabolism, in relation to adrenal cortex. Ciba Found. Colloq. Endocrinol. Vol. IV. Anterior pituitary secretion and hormonal influences in water metabolism. *London, J. & A. Churchill Ltd.* 1952. p. 442.

WEISBERG, H. F., Water, electrolyte and acid-base balance. *Baltimore, Williams & Wilkins Company.* 1955. p. 34.

VAN DER VIES, J., Two methods for the determination of glycogen in liver. *Biochem. J.* 1954. *57*. 410—416.

Acta physiol. scand. 1959. *45*. 363—368.

From the Institute of Hygiene, Karolinska Institutet, the Department of General Hygiene, the National Institute of Public Health, and the Departments of Industrial Medicine and Gynaecology, the Southern Hospital, Stockholm, Sweden.

Transplacental Diffusion of Carbon Monoxide in Human Subjects.

By

LARS FRIBERG, ÅKE NYSTRÖM
and HERBERT SWANBERG.

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Abstract.

FRIBERG, L., Å. NYSTRÖM and H. SWANBERG. Transplacental diffusion of carbon monoxide in human subjects. *Acta physiol. scand.* 1959. *45*. 363—368. — The content of carbon monoxide in maternal and in foetal blood was studied at various times after exposure to CO in concentrations sufficient to produce COHb levels of about 15 per cent in the mother's blood. CO diffused slowly from maternal to foetal blood. When exposure to CO was brief, the readings in foetal blood did not attain the levels to which the concentrations in maternal blood had fallen until 3 to 5 hours after exposure. Even when sufficient CO was breathed to maintain the maternal COHb at about 15 per cent for 2 hours, the content of CO was still not quite so high in foetal as in maternal blood. The disappearance of CO was somewhat slower from foetal blood than from maternal blood.

That carbon monoxide can diffuse from the maternal to the foetal blood stream has been reported by a number of writers. Direct measurements of CO in the blood provided the evidence

in some cases. It is also known that in the pregnant woman acute CO poisoning may cause death of the foetus although the woman herself survives. For details the reader is referred to CURTIS *et al.* (1955), who reviewed the clinical reports and animal experiments on the subject and described studies of their own on rabbits. The observations in the literature are not wholly uniform, however. Thus, no CO was found in the blood of foetuses from three women who died suddenly of CO poisoning (HELPERN and STRASSMAN 1943, MARTLAND and MARTLAND 1950), and the writers in question expressed disbelief in transplacental diffusion of the gas. CURTIS *et al.*, on the other hand, considered their own and some earlier animal experiments (DÉROBERT, BRETON and BARDON 1949) to indicate that the failure to demonstrate CO in the human foetuses was attributable to the rapid maternal death, which did not give the gas time to traverse the placental barrier. They themselves demonstrated a time lag before CO had diffused from maternal to foetal blood. GEMZELL, ROBBE and STRÖM (1957) studied the exposure of pregnant women to CO in association with normal delivery and found a time lag in diffusion of CO from maternal to foetal blood. They further stated that the foetus gradually acquired a higher COHb reading than that in the maternal blood.

In the present study which was made independently of that of GEMZELL *et al.* some observations following exposure to CO of women who underwent legal abortion on socio-medical indications are reported.

Methods.

Our aim was to observe the CO content in the blood of mother and foetus at various intervals after exposure to CO. In one type of experiment the women breathed the gas for 5 min only (brief exposure). In the second type of experiment the women were likewise exposed to CO for 5 min, but during the following 2 hours they continued to breathe CO in a concentration sufficient to maintain the level reached in the blood after 5 min (protracted exposure).

The abortions were performed by transabdominal incision under ether and nitrous oxide anaesthesia. When the exposure to CO was made during the actual operation, intravenous evipan anaesthesia was used.

Maternal blood was sampled from a median vein of the arm and, in association with the extraction of the foetus, also from a uterine vein. Foetal blood was sampled from the heart.

The CO concentration in the blood was determined according to WENNESLAND (1940). It was recalculated to COHb on the assumption

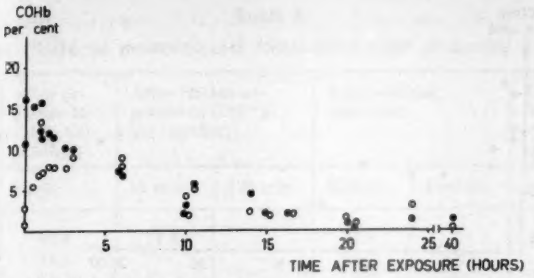

Fig. 1: *Brief exposure.* Content of CO in maternal and foetal blood at different times after 5-minute exposure.

that 1 g of hemoglobin is capable of binding 1.34 ml of CO. Double or triple measurements were made in almost all tests. The error of the method was about 0.3 per cent COHb.

Hemoglobin measurements were made with a Beckman B spectrophotometer after the principles stated by DRABKIN and AUSTIN (1936). All estimations were made in duplicate. The error of method here was about 0.2 g of Hb per 100 ml blood.

Brief exposure.

The patients were 22 somatically healthy women, most of them about 30 years old. With few exceptions, they were at the end of the fifth month or the beginning of the sixth month of pregnancy.

They were exposed to CO for 5 min with the aid of a spirometer to which was added sufficient pure CO to give a concentration of 1.5 per cent in the inhaled air. The aim was to reach about 15 per cent COHb immediately after exposure.

Two of the patients were exposed to CO during the operation. In the others the operation was performed at varying times — up to 40 hours — after termination of the CO exposure (Fig. 1). Samples of maternal and foetal blood were collected simultaneously, directly after the foetus was extracted. The mother's blood was also sampled immediately following inhalation of CO.

Protracted exposure.

The patients were 14 somatically healthy women, generally about 30 years old. With few exceptions, they were at the end of the fifth month or the beginning of the sixth month of pregnancy.

They were exposed to CO for about 5 min with the method used above. Thereafter they breathed 0.01 volumes per cent CO for 2 hours. To check that the CO concentration on the blood was maintained at the desired level (about 15 per cent COHb), the maternal blood was sampled immediately after the 5-min exposure and again after 15 min, 1 hour and 2 hours.

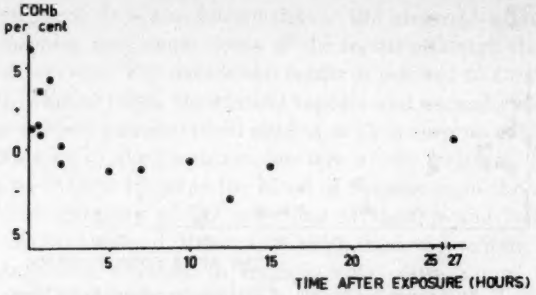

Fig. 2: *Protracted exposure.* Differences between CO content in maternal and in foetal blood. A positive difference implies higher concentration in the maternal blood.

The abortion was performed at times ranging from immediately to 27 hours after termination of CO exposure (Fig. 2). As in the experiments with brief exposure, samples of maternal and foetal blood were taken simultaneously, as soon as the foetus was extracted.

Results.

The results of brief exposure to CO are presented in Fig. 1. Immediately after exposure to CO a clear and considerable difference of CO content was found between maternal and foetal blood. The difference diminished with time and was largely eliminated after 3 to 5 hours.

The results of protracted exposure are shown in Table I and in Fig. 2. The table shows the CO content in the maternal blood during exposure to CO and in the maternal and foetal blood when the foetus was removed from the uterus. The figure shows the difference between the maternal and foetal CO readings at different times after end of exposure. To begin with this diffference was clearly positive, *i. e.*, the CO content was higher in the maternal than in the foetal blood. After 3 to 5 hours, however, the difference became negative, implying a higher concentration of CO in the foetal blood.

Discussion.

Our observations confirm earlier reports that CO can be transmitted from maternal to foetal blood. The rate of diffusion clearly is so slow that brief exposure to CO should not as a rule give rise

Table I.

Per cent COHb in maternal and foetal blood after protracted exposure.

Patient	After exposure to 1.5 % CO (mother)	After further exposure to 0.01 % CO (mother)		Values during operation		Time from cessation of CO exposure to operation
	5 min	15 min	120 min	Mother	Foetus	
1	15.0	14.8	—	13.8	7.7	0 min
2	14.4	—	—	14.7	13.3	0 »
3	11.1	10.3	12.5	9.0	7.5	35 »
4	16.2	14.0	17.5	12.6	9.0	40 »
5	13.1	12.6	13.5	10.5	9.6	40 »
6	15.2	14.8	14.5	11.8	7.5	80 »
7	13.8	13.4	13.0	8.6	9.5	2 hours
8	—	13.5	12.5	9.7	9.4	2 »
9	18.1	13.8	21.2	9.0	10.3	5 »
10	14.2	15.5	13.8	7.9	9.2	7 »
11	20.1	16.2	15.3	6.0	6.8	10 »
12	17.6	17.8	17.1	3.6	6.6	12 1/2 »
13	15.4	13.6	—	0.7	1.8	15 »
14	18.0	18.3	15.3	2.3	1.7	27 »

to COHb concentrations in foetal blood which at all approach those in the mother's blood. Thus, in our experiments, the foetal levels of CO immediately after such exposure were, with a single exception, no higher than about half of the readings in maternal blood. The maximal concentrations of CO in foetal blood were found after 3 to 5 hours, when they rose to levels equalling those to which the maternal readings had fallen. A practical deduction from these observations is that in pregnant women who have been poisoned by brief exposures to CO, early measures to flush out the gas should result in reduction of the quantity diffused to the foetus. Even without such measures, however, one may anticipate that in such a case the content of CO in the foetal blood will be of a lower order of magnitude than the level in the maternal blood.

In more protracted exposures to CO, the circumstances are different. Although in our experiments the CO concentration after 2 hours of exposure was less in foetal than in maternal blood, the difference was not great. From the practical point of view, therefore, one may assume that largely similar CO levels are reached in foetal and in maternal blood.

The experiments with protracted CO exposure showed clearly that there is a time lag also as regards excretion of CO from foetal

blood. In view of the slow foetal absorption of CO this finding was not surprising. It also seems to agree with certain results in the above-mentioned, independent study of GEMZELL, ROBBE and STRÖM (1957). We found it more remarkable that delayed foetal excretion of CO was not demonstrated in the experiments with brief exposure. One must keep in mind, on the other hand, that in the latter experiments the CO content in the foetal blood was never high. Consequently, delay in excretion may well have been less obvious than after protracted CO exposure. For practical purposes the assumption would seem justifiable that, as the result of the more rapid excretion of CO from the mother, the content in the foetal blood may gradually exceed that in the maternal blood. With the CO concentrations we used, however, the differences were small.

References.

CURTIS, G. W., E. J. ALGERI, A. J. MCBAY and R. FORD, *Arch. Path.* (Chicago) 1955. *59*. 677.
DÉROBERT, L., R. LEBRETON and J. BARDON, *Ann. Méd. lég.* 1949. *29*. 336.
DRABKIN, D. and J. AUSTIN, *J. Biol. Chem.* 1936. *112*. 51.
GEMZEL, C. A., H. ROBBE and G. STRÖM, *Acta physiol. scand.* 1957. *42* Suppl. 145.
HELPERN, M. and G. STRASSMAN, *Arch. Path.* (Chicago) 1943. *35*. 776.
MARTLAND, H. S. and H. S. MARTLAND, JR., *Amer. J. Surg.* 1950. *80*. 270.
WENNESLAND, R., *Acta physiol. scand.* 1940. *1*. 49.

Acta physiol. scand. 1959. 45. 369—374.

From the Department of Pharmacology, University of Uppsala, Sweden.

The Phagocytic Activity of the Reticulo-endothelial System in Alloxan-diabetic Rabbits.

By

LENNART JUHLIN.

Received 28 November 1958.

Abstract.

JUHLIN, L. The phagocytic activity of the reticulo-endothelial system in alloxan-diabetic rabbits. *Acta physiol. scand.* 1959. *45*. 369—374. — The functional activity of the reticulo-endothelial system in alloxan-diabetic rabbits has been measured by studying its ability to eliminate intravenously injected spherical particles from the blood stream. The distribution of the particles in different organs was also studied after they had left the blood. It was found that the particles disappeared more slowly from the blood stream in alloxan-diabetic animals than in the controls. The distribution of particles in different organs was the same in both groups. The decreased phagocytic activity of the reticulo-endothelial cells in diabetic animals may contribute to the decreased resistance to infections of diabetics.

The decreased resistance to infections of diabetic patients is well documented. The cause is still obscure, however, though several explanations have been put forward (DUNCAN 1957). In the skin, for example, the increased amount of glucose was thought to improve conditions for bacterial growth. The state of the connective tissue (JUHLIN 1956 a) and vascular and hormonal factors have also been considered.

The relationship between the function of the reticulo-endothelial system (R.E.S.) and the natural resistance to shock and infections has been discussed by several authors (JUNGEBLUT 1930, HALPERN 1957, ZWEIFACH, BENNACERRAF and THOMAS 1957, BOEHME and DUBOS 1958). I have been unable to find any report in the literature concerning the phagocytic activity of the R.E.S. in diabetes, but impaired R.E.S. function might well be a factor of importance in explaining the decreased resistance to infection of these patients.

An investigation into the phagocytic activity of the R.E.S. in alloxan-diabetic rabbits has now been made by studying the elimination of injected spherical particles from the blood stream and their distribution within the R.E.S.

Methods.

Test animals. Male albino rabbits with an initial weight of 2.0 ± 0.1 kg were used. They were fed on hay, oats, and water ad. lib.

Alloxan injection. Alloxan (Eastman-Kodak) was injected intravenously during 2—3 min in a dose of 150 mg/kg body weight, as a freshly-prepared, 5 per cent solution in distilled water. To counteract early death from hypoglycaemia, glucose was given during the first 24 hours as described by BÁRÁNY and BROLIN (1953). After 5 days and after 3—4 weeks on the day before the injection of particles urine-sugar was determined with Clinitest Reagent Tablets® (Ames Company, London). Animals with less than 0.5 % glucose in the urine on both occasions were discarded.

The alloxan animals were weighed again on the day before the experiments. Some animals had now increased in weight, and some had decreased. The mean body-weight was slightly decreased, the range now being 1.50—2.10 kg. No correlation was seen between the degree of glucosuria and change in body weight.

Particles. Solid spherical particles of methyl methacrylate, marked with a fluorescent dye, 'Fluorescent Green H.W. 185 %' (Wilmont & Cassidy Inc. N.Y.) were used. These were from the same batch as described in a previous report (JUHLIN 1956 b) in which the diameter of the particle was about 0.1 μ and their HLB (hydrophilic-lipophilic balance) value about 40. As can be seen from electron micrographs and the size-distribution table (JUHLIN 1956 b), the particles are fairly uniform in size.

Doses injected. From the original particle suspension, which contained 24 % by weight of solids, 1 ml was added to 14 ml of 5 % sterile glucose solution. Of this solution two intravenous injections of 6 ml were made at an interval of one hour. Each rabbit thus received 192 mg of solid particles.

Procedure. This has previously been described in detail (JUHLIN 1958). The suspension was injected intravenously into unanaesthetized rabbits.

One ml of arterial blood was then immediately and at regular intervals collected from an indwelling needle in the central artery of one ear, and added to 10 ml of pure acetone. After one hour the same number of particles was injected again, and blood collected as before. Three hours after the first injection the animals were killed, and certain internal organs removed and wet-weighed. The particles were found to be localized in the liver, spleen, lung, and bone marrow. The amount of particles in these organs and blood was estimated by determining the fluorescence in 0.5 g, or usually 1.0 g, of minced tissue to which 10 ml of acetone had been added to dissolve the particles and their fluorescent dye. The determinations were made in a Coleman Electronic Photofluorometer, solutions of known particle content being used as standards. In animals in which non-fluorescent particles had been injected, practically no fluorescence was found in blood, bone marrow, spleen, or lung. Some fairly constant fluorescence was found in the liver, corresponding to a mean of 168 ± 24 μg particles/g liver tissue. The figures for particle-content in the liver have been corrected for this blank value.

Phagocytic index and method of combining results of different experiments.

The clearance of particles from the blood stream takes place largely in accordance with an exponential equation $(\log C_1 - \log C_2) / (T_1 - T_2) = K$, where C_1 and C_2 are the concentrations of particles in mg/100 ml blood at times T_1 and T_2 respectively. K is a constant representing the rate of clearance of particles from the blood, and termed the *phagocytic index* (Benacerraf *et al.* 1957). The phagocytic index has been calculated both after the first and second injections of particles. If K is divided by $\log. \frac{100}{99} = 0.0044$, the percentage fall in particle concentration per minute is obtained.

The geometric means of the particle concentrations noted at certain time-intervals after injection form the basis of the average curves in Fig. 1. This ensures that the rectilinearity on semilogarithmic paper of the individual experimental curves is not destroyed by the pooling of the data.

The values for fall in particle concentration in Table I are arithmetic means of individual values obtained by plotting each individual experiment on semilogarithmic paper and visually assessing the percentual fall from the straight section of the curve. This average value corresponds closely with the percentage fall in the straight section of Fig. 1 obtained by another but equivalent arithmetical method.

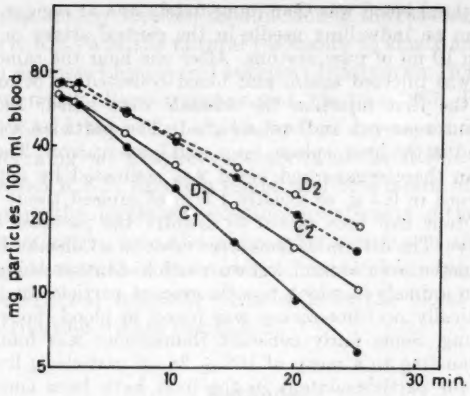

Fig. 1. Clearance of particles from the blood of alloxan-diabetic rabbits (D) and controls (C). The same dose 96 mg was given twice with an interval of 1 hour (subscripts 1 and 2).

Abscissa: Time in minutes after injection of particles.
Ordinate: Particles, mg/100 ml blood.

Results.

The results are summarized in Fig. 1 and Tables I and II. The following conclusions may be drawn.

1. The particles disappear more slowly from the blood stream of alloxan-treated animals.

2. The distribution of particles is the same as in normal animals.

Discussion.

Starvation during 4 days has been shown to decrease the phagocytic activity of the R.E.S. (JUHLIN 1958). Since some of the alloxan-treated animals decreased in body weight, and since the others did not increase as much as the control animals, the decreased phagocytic activity might have been due to undernutrition. However, no correlation could be found between the degree of change in body weight and the phagocytic activity; and the weight of the liver and spleen did not decrease as in the starvation experiments. Further, the distribution of particles between liver

Table I.

Percentage fall in particle concentration per minute in blood. The figures indicate arithmetic mean ± the standard error of the mean. The probability that the difference between controls and alloxan-diabetic animals is caused by random factors is < 0.01.

	Controls	Alloxan-diabetic animals
Injection I	10.0 ± 0.7	7.2 ± 0.5
Injection II	7.7 ± 0.7	5.4 ± 0.4

Table II.

Distribution of spherical particles in certain organs. Injected dose of particles 192 mg. All organ analysis made 2 hours after the last intravenous injection. The figures indicate arithmetic mean ± the standard error of the mean.

	Controls	Alloxan-diabetic animals
Liver; weight g	66.4 ± 5.4	60.8 ± 3.4
% of injected dose	66.9 ± 1.6	68.7 ± 2.1
Spleen; weight g	1.1 ± 0.2	1.0 ± 0.1
% of injected dose	5.8 ± 0.6	5.1 ± 0.8
Bone marrow; weight g	30.0	27.0
% of injected dose	24.8 ± 1.6	26.7 ± 4.7
Lung; weight g	9.2 ± 0.5	9.2 ± 0.6
% of injected dose	1.9 ± 0.2	0.9 ± 0.1
Body weight	2.00 ± 0.03	[1] 1.85 ± 0.10 [2] 1.80 ± 0.22
Number of animals	9	8

[1] The day before alloxan injection.
[2] The day before the experiment.

and bone marrow differs in starved animals and animals with alloxan diabetes. In animals starved during 4 days the amount of particles in the bone marrow was increased, whereas it had decreased in the liver (JUHLIN 1958). This was explained by assuming that starvation did not decrease the phagocytic activity of the bone marrow as much as that of the liver. In the present experiments the distribution of particles was the same in alloxan diabetic animals as in the controls, indicating a more even depression of the

R.E.S. in different organs. It therefore appears unlikely that the depressant effect on the R.E.S. is due entirely to starvation. In any event, the decreased phagocytic activity in diabetic animals may well be a factor contributing to their decreased resistance to infections.

Thanks are due to Mrs. ASTA PALIS for valuable technical assistance and to Dr. B. AF EKENSTAM and Mr. A. FURENDAL, A.-B. Nobelkrut, Bofors, Sweden, for preparation of the particles.

References.

BÁRÁNY, E. H. and S. E. BROLIN, Failure of Subconjunctival Injections of Insulin to Prevent Diabetic Cataract by Local Action. *Acta Soc. Med. upsalien.* 1953. *59.* 250.

BENACERRAF, B., G. BIOZZI, B. N. HALPERN and C. STIFFEL, In Physiopathology of the R.E.S. Oxford Blackwell 1957.

BOEHME, D. and R. J. DUBOS, The effect of bacterial constituents on the resistance of mice to heterologous infection and on the activity of their reticuloendothelial system. *J. exp. Med.* 1958. *107.* 523—536.

DUNCAN, G. G., Diseases of Metabolism. Philadelphia, Saunders Comp. 1944.

HALPERN, B. N., Le système réticulo-endothélial et son rôle dans la défense de l'organisme. Acquisations Médicales Récentes 1956. Paris Ed. *Med. Flammarion* 1957, p. 237—255.

JUHLIN, L., Studies on the dermal connective tissue barrier of alloxan-diabetic rabbits and its reconstitution after administration of testicular and bacterial hyaluronidase. *Acta derm.-venereol.* (Stockh.) 1956 a. *36.* 85—95.

JUHLIN, L., The spreading of spherical particles in dermal connective tissue. *Acta derm. -venereolog.* (Stockh.) 1956 b. *34.* 131—145.

JUHLIN, L., The effect of starvation on the phagocytic activity of the reticulo-endothelial system. *Acta physiol. scand.* 1958. *43.* 262—274.

JUNGEBLUT, C. W., Die Bedeutung des Reticulo-Endothelialen Systems für die Infection und Immunität. *Ergebn. Hyg. Bakt.* 1930. *11.* 1—67.

ZWEIFACH, B. W., B. BENACERRAF and L. THOMAS, The relationship between the vascular manifestations of shock produced by endotoxin, trauma, and hemorrhage. II. The possible role of the R.E.S. in resistance to each type of shock. *J. exp. Med.* 1957. *106.* 403—414.

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[1] Pr

Acta physiol. scand. 1958. 45. 375—380.

From Johan Throne Holst's institutt for ernæringsforsking, University of Oslo, Norway.

Liver Mitochondria Lipids in Hypercholesterolaemic Rats Given Polyenoic Fatty Acids.

By

JENS G. HAUGE.[1]

Received 6 December 1958.

Abstract.

HAUGE, JENS G. Liver mitochondria lipids in hypercholesterolaemic rats given polyenoic fatty acids. *Acta physiol. scand.* 1959. *45.* 375—380. — The reductions in serum cholesterol observed in this laboratory in experimental hypercholesterolaemic rats given small doses of polyunsaturated fatty acids over a period of 14 days may be due to an increase in the cholesterol degrading activity of the liver mitochondria. The effect of the treatment on the polyenoic fatty acid composition of the mitochondrial lipids was therefore investigated and correlated to the changes in total serum lipids and the serum cholesterol esters. Parallel changes in mitochondria and serum were found, linoleate affecting the dien and tetraen content, linolenate and pentaen- and hexaen-rich fractions of cod liver oil affecting the pentaenoic and hexaenoic acid concentrations. An important exception to this rule, however, was found in the high and almost constant content of trienoic acids in the mitochondria, an acid group abundantly present also in the liver mitochondria of rats on the linoleic acid containing stock diet. The probable nature of the trienoic acids involved is discussed.

[1] Present address: State Institute for Public Health, Oslo.

A reasonable hypothesis to explain the reductions in serum cholesterol observed when rats raised on a 1 % cholesterol-10 % hydrogenated coconut oil diet are given polyenoic fatty acids (HAUGE and NICOLAYSEN 1958) would be that this supplementation leads to an increased efficiency of the cholesterol degradation system. The effect of hypo- and hyperthyroidism on the serum cholesterol level is well known, and it has been demonstrated that the thyroid hormone regulates the speed of the conversion of cholesterol to bile acids (WEISS and MARX 1955, ABELL *et al.* 1956). Recent investigations have shown an increased formation and excretion of bile acids in human subjects following an administration of unsaturated acids that simultaneously lowered the serum cholesterol level (LEWIS 1958, HAUST and BEVERIDGE 1958).

The bile acid formation takes place in the liver, and within the liver tissues the mitochondria appear to be the agents most directly responsible for the process (HORNING *et al.* 1957). The mitochondrion is of lipo-protein nature, and a modification of this structure is in general accompanied by marked changes in its catalytic properties. It was therefore of interest to investigate whether supplementation for 14 days of the cholesterol-coconut diet with small amounts of polyunsaturated acids would significantly affect the mitochondrial lipids.

Experimental.

Biological. A bioassay of the cholesterol-depressive effect of methyl linoleate, methyl linolenate (Hormel Foundation), and two concentrates of cod liver oil pentaenoic and hexaenoic acid methyl esters (Professor Notevarp, Trondheim) was carried out according to the routine procedure of this laboratory. The results of this assay will appear elsewhere. From the animals used, serum samples and liver mitochondria were combined groupwise and subjected to lipid analysis. A group of the stock males, three months of age, was also included.

Chemical methods. The liver tissue was disintegrated in a teflon-pestle homogenizer in 8.5 % sucrose, and the mitochondria isolated by differential centrifugation, essentially according to SCHNEIDER (1948). Extraction of total lipids from the mitochondria was first attempted with methanol-chloroform, following the procedure used by MARINETTI *et al.* (1957) on cytochrome oxidase. Variable and low lipid contents, averaging 20 % of the dry weight were found with this method. Extraction with ethanol-ether, essentially according to

Table I.

Mitochondrial lipids.

Type of oil fed	% of total lipids					
	cholest.	dien	trien	tetraen	pentaen	hexaen
control group	8.4	0.3	8.6	2.3	1.3	3.0
methyl linoleate	8.3	1.7	7.9	4.3	0.9	0.7
methyl linolenate[1]		1.1	8.5	2.6	3.0	4.1
hexaen conc.	6.5	— 0.4	7.9	1.6	1.9	4.6
pentaen conc.	7.1	— 0.9	7.2	1.3	3.3	5.4
stock diet	3.7	3.3	5.7	7.5	1.1	3.3

[1] These data were obtained on the incomplete extract, no complete extract being available. The trienoic value has been adjusted upwards the amount expected (cf. Experimental).

HANAHAN *et al.* (1957), gave lipid yields for the experimental groups from 29.2 to 30.4 %, and 27.0 % for the stock animals. These values are in the expected range (SCHNEIDER and HOGEBOOM 1951). The polyenoic acid composition of the incomplete and the complete extract was largely the same, except for the trienoic concentration, which in average was 70 % higher in the complete extract. Extraction of total serum lipids, isolation of the cholesterol ester fraction, cholesterol determinations, and alkali isomerizations were carried out as described earlier (HAUGE 1958). A more recent formula for the computation of the polyenoic composition was, however, used (HOLMAN 1957). The k-values were determined for serum total lipid after multiplication of the weights isomerized by 0.7, so as to get an approximate measure of the polyenoic fatty acid content in % of total serum fatty acids. This factor is based on the rat serum composition given by MUKHERJEE *et al.* (1957). For the cholesterol esters the corresponding factor was 0.44 (as derived from cholesterol stearate). In the case of the mitochondria lipids the formula in two instances gave a small negative dien content (Table I). This must be ascribed to a relatively large difference in nature between the fat investigated and the reference substances on which the formula was based. The dienoic content of the total serum lipid is on the other hand somewhat overestimated, due to a variable extraneous absorption in the lower ultraviolet, that could not properly be corrected for. These phenomena serve to emphasize the semiquantitative nature of the procedure and the caution with which the results should be interpreted.

Results and Discussion.

The dietary regimen of cholesterol-hydrogenated coconut oil has an effect on the rat lipids that clearly is revealed also in the liver mitochondria (Table I). The main difference between

Table II.

Serum lipid composition.

Type of oil fed	cholest. mg%	% of total serum (s) and cholest. ester (ch) acids as									
		dien		trien		tetraen		pentaen		hexaen	
		s	ch	s	ch	s	ch	s	ch	s	ch
control group	403	1.6	3.2	1.8	2.6	0.3	0.9	0.1	0.1	0.3	0.2
methyl linoleate	309	2.0	4.8	1.4	3.4	1.4	3.2	0.1	0.3	0.1	0.2
methyl linolenate	211	4.4	2.9	2.8	2.2	1.1	1.2	1.9	2.3	0.7	0.3
hexaen conc.	205	3.1	5.5	0.4	2.9	0.4	2.1	2.0	5.6	1.1	2.0
pentaen conc.	194	2.3	4.6	1.1	3.4	0.7	1.9	2.0	6.3	0.6	1.4
stock diet	62	7.1	21.1	0.9	4.5	4.2	24.4	0	1.8	0.6	1.5

the control and the stock colony groups lies in the cholesterol content, which is doubled in the control, and in the dienoic and tetraenoic acid contents. The high dienoic and tetraenoic levels in the stock animals reflect the high linoleic acid content of the peanut oil in the stock diet. The presence of significant amounts of pentaenoic and hexaenoic acids in both groups is interesting, since the pentaens and hexaens so far isolated from mammalian livers appear to have a linolenic acid related structure (KLENK 1957). Even more striking is the high level of trienoic acid in the stock animals. The appearance of large amounts of trien has often been observed in fatty acid deficiency. KLEIN and JOHNSON (1954 a) found dien, trien, tetraen, and pentaen in the mitochondrial lipids of corn oil fed rats in concentrations 20, 1, 22, and 5 μg per mg nitrogen. In animals on a fat free diet these concentrations after 12—24 weeks changed to 8, 10, 10 and 2 μg per mg. In an earlier experiment (KLEIN and JOHNSON 1954 b) a similar increase in mitochondrial trienoic acid occurred in rats of one strain receiving 1.3 mg of linoleic acid per gram of diet for 12—16 months, while another strain receiving 2.0 mg/g showed no such increase. The authors concluded that the trien increase here also was due to essential fatty acid deficiency. Such an interpretation is not possible in the present experiment, where the stock animals had received 13 mg linoleic acid per gram of diet. The trien found in deficiency is according to MEAD and SLATON (1956) related to oleic acid, its structure being 9, 12, 15-C_{18} trienoic acid (numbered from the terminal methyl). The trien found abundant in the stock animal mitochondria in the present

study would be expected to be of a different nature, and may possibly represent the 6, 9, 12-C_{18}-trien (γ-linolenic acid) described as a probable intermediate in the formation of arachidonate from linoleate (MEAD and HOWTON 1957). This could conceivably also be the case in KLAIN's 1.3 mg/g group, if a strain-specific slowdown of the conversion of γ-linolenic to arachidonic would develop with age.

In the essentially fatty acid deficient control group of Table I the trienoic content is further elevated, and the supplementation of the basic diet with 40 mg daily for 14 days of the oils noted resulted in only slight decreases in trien. There may, however, well be a change in the relative proportions of the various possible trienoic acids. An effect of the supplementation that is discernable by the alkali isomerization technique is seen on the dien and tetraen content after linoleate feeding, and on the pentaen and hexaen concentrations after feeding of linolenate and the highly unsaturated concentrates. The mitochondria lipid picture thus in some respects appears to change in parallel to that of the cholesterol esters, described earlier (HAUGE 1958).

In order to have this correlation demonstrated within one and the same animal experiment, the total serum lipids and the serum cholesterol esters were also isolated and analyzed. The results are compiled in Table II. The expected increase in tetraene following linoleate administration is apparent, and so is the increase in pentaene and hexaene following administration of the other oils. The proportion of polyunsaturated acids in the cholesterol fraction is on an average 2.5 times that of the total serum fatty acids.

In conclusion then, it may be stated that the administration of the unsaturated acids have affected not only the composition of the cholesterol esters, and the serum lipids in general, but also the mitochondrial lipids. The changes in the polyunsaturation pattern of the serum cholesterol fraction, giving an amplified expression of the changes in the total serum lipids, also give a good indication of the degree of change in the mitochondrial tetraenoic, pentaenoic and hexaenoic acids. The partial autonomy of the mitochondrial lipid system is, however, emphasized by the high and stable total concentration of its trienoic fatty acids.

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References.

ABELL, L. L., E. H. MOSBACH and F. E. KENDALL, *J. biol. Chem.* 1956. *220*. 527.
HANAHAN, D. J., J. C. DITTMER and E. WARASHINA, *J. biol. Chem.* 1957. *228*. 686.
HAUGE, J. G. and R. NICOLAYSEN, *Acta physiol. scand.* 1958.
HAUGE, J. G., *Acta physiol. scand.* 1958.
HAUST, H. L. and J. M. R. BEVERIDGE, *IV Int. Congr. Biochem.* Abstr. 1958. 204.
HOLMAN, R. T., in "*Methods of Biochemical Analysis*". *4*. 122. (P. Glick, ed.) Interscience. New York. 1957.
HORNING, M. G., D. S. FREDRICKSON and C. B. ANFINSEN, *Arch. Biochem. Biophys.* 1957. *71*. 266.
KLEIN, P. D. and R. M. JOHNSON, *Arch. Biochem.* 1954 a. *48*. 380.
KLEIN, P. D. and R. M. JOHNSON, *Arch. Biochem.* 1954 b. *48*. 172.
KLENK, E. and H. J. TOMUSCHAT, *Z. physiol. Chemie.* 1957. *308*. 165.
LEWIS, B., Lancet 1958. *i*. 1090.
MARINETTI, G. V., D. J. SCARAMUZZINO and E. STOTZ, *J. biol. Chem.* 1957. *224*. 819.
MEAD, J. F. and D. R. HOWTON, *J. biol. Chem.* 1957. *229*. 575.
MEAD, J. F. and W. H. SLATON, Jr., *J. biol. Chem.* 1956. *219*. 705.
MUKHERJEE, S., K. T. ACHAYA, H. J. DEUEL, Jr. and R. B. ALFIN-SLATER, *J. biol. Chem.* 1957. *226*. 845.
SCHNEIDER, W. C., *J. biol. Chem.* 1948. *176*. 259.
SCHNEIDER, W. C. and G. H. HOGEBOOM, *Cancer Res.* 1951. *11*. 1.
WEISS, S. B. and W. MARX, *J. biol. Chem.* 1955. *213*. 349.

220. 527.
228. 685.

1958. 204.
lick, ed.)

. Biophys.

1957. 224.

ER, J. biol.